CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol. V. Bioassay of Anterior Pituitary and Adrenocortical Hormones

Other Volumes in this Series

Vol. I	Steroid Hormones and Tumour Growth *and* Steroid Hormones and Enzymes	30s.
Vol. II	Steroid Metabolism and Estimation	35s.
Vol. III.	Hormones, Psychology and Behaviour *and* Steroid Hormone Administration	35s.
Vol. IV	Anterior Pituitary Secretion *and* Hormonal Influences in Water Metabolism	45s.
Vol VI.	Hormonal Factors in Carbohydrate Metabolism	*Ready Spring* 1953
Vol. VII.	Synthesis and Metabolism of Adrenocortical Steroids	*Ready Spring* 1953

CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME V

Bioassay of Anterior Pituitary and Adrenocortical Hormones

General Editor for the Ciba Foundation

G. E. W. WOLSTENHOLME, O.B.E., M.A., M.B., B.Ch.

Assisted by

MARGARET P. CAMERON, M.A., A.B.L.S.

With 53 Illustrations



LONDON

J. & A. CHURCHILL LTD

104 GLOUCESTER PLACE, W 1

1953

All Rights Reserved

This book may not be reproduced by any means, in whole or in part, without the permission of the Publishers

Printed in Great Britain

PREFACE

THE Ciba Foundation, an Educational Charity under English law maintained by Ciba Limited of Switzerland and administered exclusively by its distinguished Trustees (Dr. E. D. Adrian, OM, FRS, Lord Beveridge, KCB, FBA, Lord Horder, GCVO, and Mr. Raymond Needham, QC), was formally opened by Sir Henry Dale in 1949. It provides in its house in London an international centre where those engaged in medical and chemical research may stay during visits connected with their work, and where they may take part in meetings of a few hours to a few days in duration on any subject from the whole territory of biological research. Of the longer, international meetings, attended by invitation, there have been 19 in the first three years of the Foundation's active existence.

In all these conferences an attempt has been made to secure a "house-party" atmosphere, where frank and informal exchanges of information and opinion on current work have been encouraged rather than the formal announcement of completed work, more suitably presented at the larger congresses. The Trustees and Director of the Ciba Foundation have been urged in every case, however, to make available the proceedings, consisting of brief programme papers followed by general discussions, and these are being assembled into volumes with only a minimum of editing in order to pass on the benefits of these small group discussions to a wider audience. In addition to much stimulating argument and speculation, the volumes contain valuable information about recent and unpublished work and many references to published papers scattered throughout the world's literature.

For the practical reason of easing the Director's task in organizing these conferences, most of them have been within one field of research, namely, endocrinology. This field was chosen because it seemed to afford a good opportunity for

the use of the Foundation as a "laboratory for mixing scientists"—Lord Beveridge's description of the Foundation—not only on an international but also on an interdisciplinary basis.

Twelve "Colloquia on Endocrinology," as they have been called, have so far been held. A summary of one, on Nomenclature of Steroids, has been published elsewhere; of the other eleven, eight have been published in four volumes, and the remaining three are being issued separately. The present volume is the first of these three to be completed; the others, on Hormonal Factors in Carbohydrate Metabolism, and Synthesis and Metabolism of Adrenocortical Steroids, will be available shortly.

The informal colloquium here recorded brings within a small compass the work of widely-separated groups studying common problems of assay of pituitary and adrenal hormones. We believe that their papers and discussions, whilst revealing much work still to be done, will prove of the greatest interest to all clinicians who need to assess the value of hormonal assays, and to all investigators who have charge of research into their greater accuracy, specificity and clinical usefulness.

As on other occasions in this series, the Editors are much indebted to Miss N. Bland for her patience and skill in obtaining recordings of the discussions, and the senior Editor for the Ciba Foundation wishes it to be known that Miss M. P. Cameron has had more responsibility for the preparation of the material than is fairly indicated by the word "assisted" on the title page.

It is with much pleasure that the Editors record their gratitude to Mr. J. Rivers and Mr. J. A. Rivers of J. & A. Churchill Ltd., whose continuous attention and kindly assistance in the publication of all the Ciba Foundation's volumes greatly eases the burden of editorial work.

G.E.W.W.

CONTENTS

PAGE

Part III—Gonadotrophins and Prolactin

PAGE

Part III—Gonadotrophins and Prolactin

Part VI—Adrenocortical Hormones

List of those participating in or attending the Colloquium on Bioassay of Anterior Pituitary and Adrenocortical Hormones, 25th to 27th March, 1952

E. B. ASTWOOD	New England Center Hospital, Boston, Mass.
R. I. S. BAYLISS	Postgraduate Medical School of London
S. W. BIBILE	Pharmacological Laboratory, University of Edinburgh, and University of Ceylon
I. E. BUSH	National Institute for Medical Research, London
W. R. BUTT	Department of Clinical Endocrinology, United Birmingham Hospitals
G. A. H. BUTTLE	Department of Pharmacology, School of Pharmacy, *University of London*
R. K. CALLOW	National Institute for Medical Research, London
D. B. CATER	Department of Pathology, University of Cambridge
PAMELA M. CLARKE	National Institute for Research in Dairying, Reading
C. L. COPE	Postgraduate Medical School of London
CONSTANCE DE COURCY	Department of Chemical Pathology, King's College Hospital, London
C. P. COX	National Institute for Research in Dairying, Reading
A. C. CROOKE	Department of Clinical Endocrinology, United Birmingham Hospitals
MARGARET L. DEDMAN	Endocrine Unit, The London Hospital, London
H. B. VAN DYKE	Department of Pharmacology, Columbia University, New York, and National Institute for Medical Research, London
E. FINGL	Pharmacological Laboratory, University of Edinburgh, and University of Utah
S. J. FOLLEY	National Institute for Research in Dairying, Reading
T. RUSSELL FRASER	Postgraduate Medical School of London
J. H. GADDUM	Pharmacological Laboratory, University of Edinburgh
I. C. GILLILAND	Postgraduate Medical School of London

C. H. GRAY	Department of Chemical Pathology, King's College Hospital, London
G. W. HARRIS	Institute of Psychiatry, Maudsley Hospital, London
J. R. HODGES	Department of Pharmacology, School of Pharmacy, University of London
D. J. INGLE	Research Division, The Upjohn Co., Kalamazoo, Mich.
J. P. JAMES	Ruakura Animal Research Station, New Zealand
C. H. LI	Department of Biochemistry, University of California
J. A. LORAINE	Clinical Endocrinology Research Unit, University of Edinburgh
N. F. MACLAGAN	Department of Chemical Pathology, Westminster Hospital Medical School, London
A. STUART MASON	Endocrine Unit, The London Hospital, London
JOYCE D. MATTHEWS	Department of Clinical Endocrinology, United Birmingham Hospitals
F. MORICARD	Centre de Stérilité de la Clinique Gynécologique, Hôpital Broca, Paris
R. MORICARD	Laboratoire d'Hormonologie, Hôpital Broca, Paris
C. J. O. R. MORRIS	Endocrine Unit, The London Hospital, London
PEGGY MORRIS	Endocrine Unit, The London Hospital, London
D. H. NELSON	U.S. Naval Medical School, Bethesda, Md.
J. A. NISSIM	Department of Pharmacology, Guy's Hospital Medical School, London
DELPHINE M. V. PARROTT	Clinical Endocrinology Research Unit, University of Edinburgh
W. L. M. PERRY	National Institute for Medical Research, London
E. REID	School of Biochemistry, University of Cambridge
A. M. ROBINSON	St. Bartholomew's Hospital, London
H. J. ROBINSON	Merck Institute for Therapeutic Research, New Jersey
J. M. ROBSON	Department of Pharmacology, Guy's Hospital Medical School, London
I. W. ROWLANDS	Wellcome Veterinary Research Station, Frant, Kent
E. F. SCOWEN	St. Bartholomew's Hospital, London

ALBERT SEGALOFF	Alton Ochsner Medical Foundation, New Orleans
ANN SEGALOFF	Alton Ochsner Medical Foundation, New Orleans
G. K SMELSER .	Columbia University, New York
M. P. STACK-DUNNF .	School of Biochemistry, University of Cambridge
MARTHE VOGT	Pharmacological Laboratory, University of Edinburgh
F G YOUNG . .	School of Biochemistry, University of Cambridge

FOREWORD

by

J. H. GADDUM, SCD, FRS

THE work of the clinical endocrinologist would be simpler than it is if it were possible to make reliable estimates of the concentrations of the various hormones in the body fluids, and much effort is being spent in the search for methods of doing this. The task is difficult because the amounts present are very small, and because accuracy can only be achieved with much labour and at vast expense. Chorionic gonadotrophin can be accurately assayed in the blood or urine of a pregnant woman, but none of the other hormones is in quite the same position. If all the claims that have been made are true, then estimates have been made of the concentrations of most of the hormones in human blood, but those concentrations are usually so low that the result is open to doubt. The time has not yet come when the physician is justified in accepting estimates of hormones without a critical consideration of the methods by which these estimates have been obtained.

The conference reported here was devoted to the consideration of assays of the hormones of the anterior pituitary and the adrenal cortex. Other hormones, such as insulin and those contained in the posterior pituitary, were not considered, but there was no lack of material for discussion and it was evident that the problem is being attacked with vigour in many parts of the world and that there is good reason to expect that bioassays will soon become less expensive and more informative.

REQUIREMENTS FOR CLINICALLY USEFUL ENDOCRINE BIOASSAYS

ALBERT SEGALOFF

WHEREAS it is easy to set up requirements for an ideal type of clinical bioassay procedure, it is extremely difficult, if not impossible, to find an assay which will fulfil even not too stringent criteria. It appears, therefore, to be advantageous to employ an allegory.

In a land of make-believe a scientist was preparing a vaporizer for use in a flame photometer, and a house fly

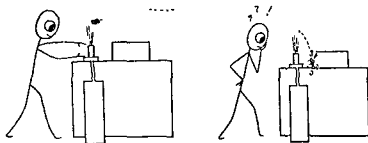


FIG. 1.

chanced to pass through a cloud of vapour produced from human serum (Fig. 1). Much to the observer's astonishment, the fly shed his wings and plummeted to the table. The scientist then captured some additional samples of *Musca domestica* and repeated what now became an experiment. Invariably, when a fly flew into the vapour produced by this sample of human serum, it immediately shed its wings and fell to the table.

Our biologist then borrowed the tools of the entomologist, namely, the breeding and care of flies. These flies were

confined, in carefully counted numbers, in cages such as those used for the assessment of insecticides. Our scientist then undertook an exhaustive series of tests, eliminating gradually all the various constituents of serum. He was particularly interested in the hormonal agents. He found that with the exception of Hormone A none of the known hormonal products produced this startling phenomenon. Thus he had

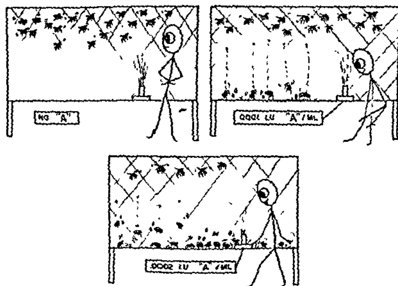


FIG 2.

proof that this was a highly specific biological reaction produced only by Hormone A. It was next necessary to evaluate the assay for its quantitative validity. He found that the percentage of flies which shed their wings was proportional to the concentration of A in the solution being vaporized (Fig. 2), and that this assay had a lambda value of 0·100.

Our scientific wizard next quickly wrote to several of his colleagues in distant lands, who upon repeating his assay procedure, obtained exactly the same results as he had.

Therefore the procedure was reproducible in other laboratories. Thus this would appear to be an assay which possesses all the attributes necessary for an ideal bioassay. To leave this Utopia and to face reality, let us now consider these qualities.

I will now list the requirements which I believe must be met by the ideal assay:—

I. Specific
II. Reproducible from laboratory to laboratory.
III. Sensitive.
IV. Statistically sound.
V. Simple.
VI. Rapid.
VII Inexpensive.

Because of the small amounts of hormonally active principles available from clinical material, sensitivity is the prime requisite. The procedure must also be specific, that is, it must measure the activity or material in question Such an assay not only will have statistical validity in one laboratory but it would be possible to reproduce the results in other laboratories It should, in addition, be simple, rapid and inexpensive.

Most clinically useful bioassay procedures begin with collection of urine Unfortunately, as far as their usefulness is concerned, they also often end here. It is amazing how many physicians think that all endocrinological diagnoses can be made on a poorly collected, haphazardly refrigerated, 24-hour urine specimen Obviously no assay can possibly be better than the urine collection on which it is started. Firstly, the patient should be adequately instructed. The urine must then be carefully refrigerated, with a preservative added when indicated, and above all a creatinine determination should always be done to check against gross errors in collection. Moreover, one must remember that the patient may be taking some medication which might interfere either by augmenting or depressing the biological assay. For

example, œstrogen medication will alter the œstrogen excretion, and in sufficient amounts will alter the excretion of gonadotrophic hormones. Further, it is not always realized that many medications, such as the sulphonamides, are excreted in forms which are toxic to adrenalectomized animals and that it is difficult to carry out a biological assay for glycogenic corticoid activity on the urine of these patients. Actually, of course, none of our available bioassays measures up to the ideal described in the introductory paragraphs. Therefore, let us consider some of the compromises which have been necessary in the development of clinically useful bioassays

In order to do this properly we must know what information we want to obtain from an assay. Do we want to be able to give a precise diagnosis (e.g., hypersecretion of glycogenic corticoids secondary to hypersecretion of ACTH), or do we want to be able to say that a patient must be treated with cortisone because she will not respond to ACTH? Whereas the former is useful clinically, the latter is adequate.

Sensitivity is fundamentally the hurdle in attempting to apply assays clinically. For example, it would probably be ideal to use some sort of assay employing humans, since there may be some species specificity to the hormones which exist in man. However, I know of no instance in which it has been possible to accomplish this for endocrinological materials of human origin The real difficulties in sensitivity, I think, can best be illustrated by the problem of the assay of glycogenic corticoid activity Such assays on a clinically useful level have been accomplished only in adrenalectomized mice. These animals show a more variable response but are more sensitive than adrenalectomized rats. Despite this, additional devices, such as pre-feeding or fortification of the extracts with glucose, are necessary in order to make the assay procedure sensitive enough so that it can be applied to the urine of normal subjects.

We are all aware of the existence of at least two, and probably a third, gonadotrophic hormone. However, with

the amounts existing in urine, it is difficult to quantitate all these by present methods of bioassay. Indeed, although we have been most interested in the assay of urinary prolactin activity, in man prolactin has not yet been definitely demonstrated to be luteotrophic, though data are accumulating which would suggest this.

Our assay for prolactin, which will be discussed more thoroughly later in this conference, may be used to illustrate one of the real difficulties which often must be overcome. This is the existence of interfering substances in the material to be used for assay The local injection of prolactin over the crop sac of the pigeon is probably the most sensitive assay for this material. However, almost all extracts which we have been able to concentrate from urine have had a distressing and severe local reaction when handled in this manner. The reaction resembles fat necrosis and interferes with the reading of the end point of the assay. It also appears to modify absorption of the hormone from the site of injection. In our experience the only way to overcome this has been to employ the much less sensitive intravenous route. Unfortunately, this assay falls short of the ideal in other respects. It is expensive, time consuming and difficult.

Of what value are bioassays in clinical medicine? An example of their usefulness might be cited here. At present, the best method of evaluating testicular failure is by use of measurement of urinary gonadotrophic hormone. Fortunately, with an intact pituitary, removal or absence of the gonads not only has an effect on the excretion of gonadal hormone but also causes the pituitary to secrete a substantially increased amount of gonadotrophic hormone, which spills over in the urine. Clinically, patients with hypogonadism and no other endocrinological abnormality present the same appearance, whether the failure is in the pituitary or the gonads. Fortunately, it is possible to differentiate these on the basis of their urinary excretion of gonadotrophic hormone. When the failure is in the pituitary, there is usually a less than normal amount of gonadotrophic hormone in the urine. In

striking contrast to this, with testicular failure or absence, there is usually an increase over the normal amount of gonadotrophic hormone in the urine. Thus the differential with respect to urinary gonadotrophic hormone between the patient with pituitary failure and gonadal failure is much greater than the difference between normal and lowered amounts of gonadotrophic hormone. Therefore, by means of bioassay procedures it is possible to separate patients with gonadal failure into two large groups. It is particularly important, for clinical purposes, to make this differentiation. In cases of testicular failure only testosterone replacement therapy is possible. However, if testosterone is given to the patient with pituitary failure, then it is impossible to obtain normal gonadal function In addition, we have the advantage, based on clinical observations, that in suitable patients with pituitary failure, administration of chorionic gonadotrophic hormone will often initiate a chain of events whereby the patient's own pituitary will be able to take over and continue secreting, so that a normal pituitary-gonad axis is maintained without the necessity of continuation of the substitution therapy. This, then, is an illustration of the major advantage which can be gained through proper utilization of bioassays.

It cannot be urged too strongly that despite the importance of bioassay procedures to the astute clinical endocrinologist, medicine is still an art, and no amount of laboratory studies can replace an intelligent and thorough history and physical examination. If this were no more than a saving of time and money, it would still be worth while. One might with profit speculate upon the large amount of money, time, and blood required to perform all the "standard" diagnostic procedures on a patient. For example, in our own institution if a patient were to have all the routine blood chemical determinations (omitting everything which could be considered as unusual), it would require 168 ml. of blood and several hundred dollars.

Thus, to return to the endocrine bioassays; after completion of a history and physical examination on a man with

hypogonadism, the assay of urinary gonadal stimulating hormone will usually make the final differential diagnosis. This can be supported by ketosteroid and androgen determinations.

Of the assays which are now needed, one for growth hormone should be listed first. Problems of growth are encountered frequently, and at present there is no real way to determine whether or not they are due to alterations in effective levels of growth hormone Moreover, there has been little or none of the expected results from therapy with excellent preparations of growth hormone in what should be adequate amounts.

Another useful assay which is needed is one of circulating ACTH on a clinical level. Published reports, particularly here in Great Britain, indicate that perhaps soon such a clinically useful procedure may be available

Finally, an effective assay for parathyroid hormone would, in all probability, facilitate the task of evaluating parathyroid function

It is hoped that data presented at this conference will point the way to bioassays previously not available

DISCUSSION

[illegible]

œstrogen. The ovarian hyperæmia method end point is also more difficult to read and, therefore, more variable

LORAINE· Would you consider the mouse uterine weight method qualitative or quantitative? Can it be put on a quantitative basis?

SEGALOFF. I would call it "semi-quantitative."

GADDUM How about the reliability of the tests for other hormones?

SEGALOFF. I think the estimation of glycogenic corticoids is useful. It is sometimes impossible to differentiate between Addison's disease [illegible] s of formal- [illegible] deposition

[illegible] t consistent results from the formaldehydogenic assay.

SEGALOFF I don't know which value to believe. With unhydrolysed urine you get one value, with enzyme-hydrolysed another, and urines

differ in their response to standing at pH 1 for 24 hours (the usual mild hydrolysis employed!). It also depends on which enzyme you use. According to some papers the ratio of the unhydrolysed value to the enzyme-hydrolysed value was stated to be of diagnostic value but in other papers this was not so. Mildly hydrolysed urine (24 hours at pH 1) is employed most widely.

BAYLISS: On the wards, it is very difficult to collect a 24-hour urine specimen free of bacterial contamination. This alone may lead to some hydrolysis. Under normal circumstances is it right, therefore, to speak of unhydrolysed urine?

SEGALOFF: I agree that this happens, but how are we to draw the line? We try to use specimens which are refrigerated immediately upon voiding. The great difficulty with enzyme hydrolysis at present is that everybody does it differently. Bacterial β-glucuronidase is different from mammalian β-glucuronidase, and then there are differences of pH, etc. Many papers do not give sufficient data to permit duplication of methods.

FRASER: For some time I have felt sceptical about the principle of 24-hour values. In using them we ignore the patients' basal metabolism. It seems to me that if we collected a single sample in the morning (8 hours' excretion) in a clean dish the problem of bacterial infection would also not arise.

SEGALOFF: It wouldn't work for the glycogenic assay of corticoids. There isn't enough material in an 8-hour urine.

FRASER: You could use two lots of night urine if that's your problem solely.

SEGALOFF: I think the diurnal variations must be eliminated by inte-

parison with other data difficult or impossible.

FRASER: But isn't this just habit? Perhaps there would be more significance if there were no diurnal variations, if the urines were obtained under basal conditions. We need to discover how much basal conditions matter to the rate of excretion.

menopause there is an increase in the secretion of hypophyseal gonadotrophin because there is an increase in the urinary excretion of gonadotrophin; it may be a loss of response of the ovaries. Clinical bioassay [illegible]

constant results.

Robson: Have you any experience of the assay of progesterone in blood? Of the Hooker-Forbes technique?

Segaloff: I haven't done it. It is a difficult assay, but I agree it would be most useful to apply. It has a very real possibility of working

Gaddum: Does the same apply to the D'Angelo technique for thyrotrophin?

Segaloff: I have avoided this because there are many difficulties. There is the problem of so-called inactivated thyrotrophin; and also the slope of the curve is very low and large increases of dosage yield small increases in response.

Fingl: You stated that your laboratory routinely does creatinine determinations on the 24-hour urine samples. Would you expand on the use or benefit of these determinations? Does superimposed renal pathology prejudice the conclusions or limit the value of the urinary assays in any other way?

Segaloff: This question of renal effect is most difficult when one tries to interpret assays on urines. However, it must also be taken into account when interpreting blood assays I don't hold a large brief for creatinine. Even in metabolic wards where there is a standard diet there are substantial variations in the values It is most useful in indicating in which samples there may be gross errors of collection. We tried expressing our results per gram of creatinine, but this wasn't too useful [illegible]

Segaloff: I agree.

THE BIOASSAY OF THYROTROPHIC HORMONE IN THE HYPOPHYSECTOMIZED RAT USING ^{32}P

M. L DEDMAN, A. STUART MASON, P. MORRIS and C. J. O. R MORRIS

THE bioassay of thyrotrophic hormone (TSH) has depended, until recently, on various methods of estimating its effect on the histology of the thyroid. The introduction of radioactive iodine has provided a means of assaying TSH in terms of thyroid function. Various techniques are now being developed to utilize this isotope as an index of thyroid activity after administration of TSH. Although the formation of thyroid hormone is intimately connected with iodine, it has also been shown (Borell and Holmgren, 1949) that changes in phosphorus metabolism occur in the thyroid when TSH is administered. These changes can be detected by the use of radioactive phosphorus (^{32}P). We have used this isotope in experiments designed to confirm and extend Borell's original work This paper is a preliminary report of our results.

It has been known for many years that phosphorus plays a part in thyroid physiology. The presence of phosphorus in the thyroid was reported first by Oswald in 1899. Kocher (1902) stated that the phosphorus content of the thyroid was inversely proportional to the iodine content. The relationship of thyroid histology to its phosphorus content was described by Aeschbacker in 1906. He found that large amounts of phosphorus were present in glands with scanty colloid, increased cellularity and many nuclei.

After this promising start interest in the subject appears to have waned until 1945 when Borell found that the phosphorus content of the guinea pig thyroid was increased after injection of TSH. He showed that the increase in total phosphorus

ran parallel to the increase in cell height. As was to be expected from this, the amount of phosphorus per unit weight of dried gland did not increase after TSH administration Borell and Holmgren (1949) then investigated the uptake of radioactive phosphorus by the guinea pig thyroid, and found that injection of TSH resulted in a marked increase in uptake. This proved to be a more sensitive response to TSH than changes in cell height. From these experiments they concluded that the ^{32}P uptake by the thyroid provided a method of assaying TSH.

Borell did not provide sufficient data in his paper to clarify the various factors which might affect his method of assay. We determined to explore these factors, and to attempt to put this assay method on a firm basis To do this it was necessary to use hypophysectomized animals, as the presence of a functioning pituitary in the experimental animal makes it difficult to assess the specificity of any assay for pituitary hormones

We have used only the hypophysectomized rat in this work; other methods of assay have shown this animal to be less sensitive than the chick to TSH, but definitely more sensitive than the intact rat. This may be due to the greatly decreased rate of TSH destruction in the rat following hypophysectomy (D'Angelo, 1951).

The ^{32}P we used was supplied free from ^{31}P. As the *p*H of the original solution was less than 2·0, it was diluted before use with an acetate buffer solution (*p*H 6·4) Each rat was injected intraperitoneally with a dose of 15 μc. of ^{32}P in a volume of 0·5 ml This dose was found to give thyroid ^{32}P levels which were convenient for counting purposes. Forty minutes after injection the rat was killed, and the thyroid dissected out. The amount of ^{32}P present in each gland was measured under constant geometric conditions with a standard Geiger counter and scaling unit. For each experiment a standard solution was prepared from the ^{32}P used for injection. The activity of the standard was determined under constant conditions using the same Geiger counter as was employed for the thyroids. For ease and

accuracy, the geometric conditions for counting the standard were not identical for these and for the thyroids. Consequently the uptake of ^{32}P by the thyroid could not be expressed as a percentage of the dose administered. The uptake, therefore, has been expressed in arbitrary units by dividing the counts per second recorded for the thyroid by the counts per second recorded for the standard, the result being multiplied by 1000. The uptake so expressed was adjusted for weight of the thyroid, when required, by calculating it as uptake per 10 mg. thyroid tissue.

The animals selected for the assay were female Wistar rats. It became apparent that both age and weight at hypophysectomy were important factors in the uptake of ^{32}P by the thyroid. Animals selected by weight regardless of age were found to have a greater variation in ^{32}P uptake than those selected for comparable age and weight. An age of 6–7 weeks at hypophysectomy was found to be convenient. Younger rats (4–5 weeks at hypophysectomy) were slightly more sensitive to TSH, but the difference in sensitivity was not marked, and the difficulties of operation greater. The weight of the rats at operation was kept between 100 to 120 g. An increase in the range of body weight led to an increase in the variation of thyroid ^{32}P uptake. In particular, rats weighing over 130 grammes showed a marked variability in ^{32}P uptake.

Our first experiments were designed to show the changes in phosphorus uptake following hypophysectomy. The uptake would be expected to decrease after operation if it was related to the action of TSH. Such a decrease did occur, as shown in Table I.

Table I

EFFECT OF HYPOPHYSECTOMY

	Thyroid wt	*Uptake**	*Uptake/10 mg*
Intact . .	8 42	353 4	421 8
5th day . .	6 8 (81%)	143 (40%)	214 (49%)
9th day .	5 7 (68%)	131 (37%)	237 (56%)
12th day .	5 29	132	256

*Arbitrary units—see text.

The drop in uptake is almost maximal by the fifth day after hypophysectomy, as compared with the decrease in gland weight. It is interesting that the drop in iodine concentration by the thyroid is maximal about the same time after operation (Randall and Albert, 1951). The table shows an apparent rise of phosphorus uptake per unit weight of gland by the twelfth day after operation, but the number of rats in this group was small, so that the finding is probably without significance.

However, the relationship between phosphorus uptake of the thyroid and its weight is not direct In the intact rat there is a significant correlation between weight and phosphorus uptake. Five days after hypophysectomy the relationship is on the borderline of statistical significance, and by the ninth day there is no correlation between weight and phosphorus uptake

The sensitivity of the thyroid to TSH at various times after hypophysectomy has not yet been fully investigated. A comparison of sensitivity at the fifth and ninth day after hypophysectomy did not show any significant difference in response to TSH. The variance in the control animals was the same at the fifth as at the ninth day, but the variance in the groups injected with TSH was greater on the ninth day. A further disadvantage of using rats on the ninth day after operation was the degree of thyroid atrophy. Therefore rats on the fifth day after hypophysectomy were chosen for assay purposes.

Having standardized the time elapsing after hypophysectomy we determined the optimum time elapsing between injection of ^{32}P and removal of the thyroid. The variation of ^{32}P uptake with time after injection is illustrated in Table II

TSH was given in four doses over the two days prior to the experiment. The maximum difference in phosphorus uptake between the control and injected groups was found at 40 minutes after injection of ^{32}P In the group given TSH there was a definite decrease in uptake by 160 minutes, presumably

Table II

VARIATION IN ^{32}P UPTAKE WITH TIME AFTER INJECTION

Time after injection (min.)	*Control*		*TSH*		$\frac{U-U_c}{U_c} \times 100$	
	U	*U/Wt*	*U*	*U/Wt.*	*U*	*U/Wt.*
40	138	221	305	421	121	91
80	157	257	324	437	116	70
120	152	231	304	376	100	63
160	163	233	263	375	61	64

U = Uptake (arbitrary units)
U/Wt = Uptake per 10 mg. thyroid tissue
U_c = Uptake for uninjected controls

indicating a more rapid turnover in phosphorus than the control group

The TSH used for assay purposes has been prepared according to the method of Ciereszko (1945) taken to stage C.A. The dose of TSH was injected subcutaneously, and two different schemes of dosage have been used. The first scheme was to divide the dose into four injections given twice daily for the two days prior to the assay. The second scheme was to give the dose in one injection four hours before injection of ^{32}P. The assay data shown in Fig. 1 compare the two schemes of dosage. For the animals injected with TSH the ^{32}P uptake has been recorded as the difference from control uptake expressed as a percentage of the control level. This has been found to be a more satisfactory method than recording the actual uptake, because there appears to be some seasonal variation in the control levels. This method of expressing the uptake eliminates apparent changes in the slope of response which are due to this seasonal change

The ratio of the slope of the response-log dose curve to the variance in the groups of rats is such that four or five rats per dose level are sufficient for assay purposes. It will be seen that four injections over two days give a response which is linear over a wider range of dosage than the single

injection assay; but the latter method is more sensitive, and gives a steeper slope.

All the data in Fig. 1 are calculated regardless of thyroid weight. Fig. 2 shows the same data expressed as uptake per 10 mg. thyroid tissue. It is apparent that this method of calculation makes the response to four injections of TSH very

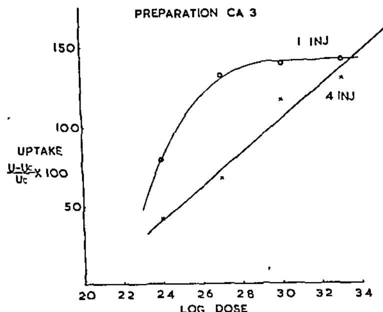


Fig 1 Effect of frequency of TSH injections on ^{32}P uptake

similar to that of the one-injection technique expressed regardless of thyroid weight. From this we concluded that the initial rise in ^{32}P uptake after TSH is independent of growth of the thyroid, but this mechanism soon reaches its maximum response. Further uptake becomes apparent with larger doses of TSH when the dosage is so spaced that the gland has time to increase its cell height. This hypothesis

s supported by data obtained from rats given TSH on the ninth day after hypophysectomy. In the control groups and those given small doses of TSH in four injections there is no correlation between ^{32}P uptake and weight of thyroid. With higher doses of TSH the correlation between uptake and weight is significant.

A comparison of the slopes of response to TSH preparations

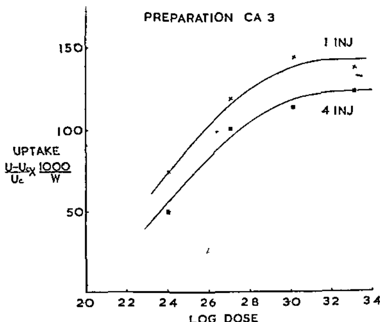


FIG. 2. Effect of frequency of TSH injections on ^{32}P uptake. Uptake values corrected for thyroid weight.

of different potency has been obtained by using a preparation of the Armour Laboratories (No. 317-51). Fig. 3 shows that the slope for this preparation is approximately parallel to that for the Ciereszko preparation. In both assays the TSH was given in four injections.

We have not as yet tested the *specificity of the assay* method by using all the other pituitary hormones available

in a purified state. However we have given 2 i.u of ACTH/rat without causing any change in ^{32}P uptake by the thyroid. It is interesting to note that the uptake of ^{32}P by the adrenals was doubled after ACTH administration.

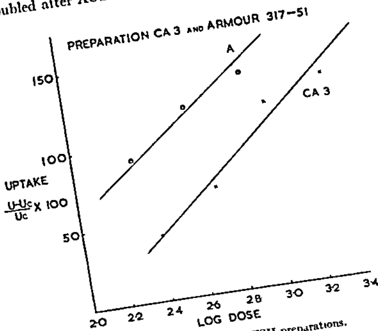


FIG 3 Comparative assay of two TSH preparations.

We have also attempted to relate the uptake of ^{32}P to various changes in thyroid function induced by anti-thyroid agents in intact rats. Table III illustrates the results.

Table III

^{32}P UPTAKE IN INTACT RATS GIVEN ANTI-THYROID AGENTS

	Control	Thyroxine	P Thio	KSCN
	100	24	216	160
	100	136	54	—

Thyroxine 20 μg. daily for 14 days decreased the uptake of ^{32}P by the thyroid, and increased the muscle uptake of ^{32}P. This ruled out the possibility that ^{32}P uptake by the thyroid was due to the stimulating effect of circulating thyroxine, and suggested that, in view of the known suppressive effect of thyroxine on TSH production, the uptake was closely related to TSH action. A similar experiment using 10 mg. of propylthiouracil daily resulted in a marked increase in thyroid uptake of ^{32}P. Thus the uptake was not dependent on formation of thyroxine (inhibited by propylthiouracil) but on the greater amount of TSH which is probably released when animals are treated with this antithyroid substance. The decrease in muscle uptake was probably due to lack of circulating thyroxine. Finally, the iodine trapping mechanism of the thyroid was inhibited by a dose of 100 mg. KSCN/day for 14 days. This led to an increased uptake of ^{32}P by the thyroid, probably for the same reason as suggested for propylthiouracil. We consider that these experiments indicate that the uptake of phosphorus by the thyroid is not related directly to the iodine cycle in the action of thyroxine. It appears that it is related to the process of cell division directly stimulated by TSH.

The observations reported form a preliminary survey, in which we have attempted to explore the variable factors concerned in the assay of TSH by ^{32}P uptake of the thyroid. At this juncture we consider that the method offers a direct measurement of thyroid response to TSH. The sensitivity of the method is limited by the choice of the rat as the experimental animal, but it is of fundamental importance to establish the basis of the assay using hypophysectomized animals We conclude that the method is promising and provides a means not only for assay for TSH but also for investigating thyroid physiology and the action of antithyroid agents.

REFERENCES

AESCHBACKER, S. (1906) *Mitt. Grenzeb. Chir.*, 15, 269.
BORELL, U. (1945). Thesis (Stockholm).

BORELL, U., and HOLMGREN, H. (1949). *Acta endocrinol.*, **3**, 331.
CIERESZKO, L. S. (1945). *J biol. Chem.*, **160**, 585.
D'ANGELO, S. A. (1951). *Endocrinology*, **48**, 249.
KOCHER, A. (1902). *Mitt. Grenzgeb. Chir.*, **9**, 1.
OSWALD, A. Z. (1899). *Physiol. Chem.*, **27**, 14.
RANDALL, R. V., and ALBERT, A. (1951). *Endocrinology*, **48**, 327.

ASSAY OF THYROTROPHIC HORMONE BY ^{131}I DISCHARGE

I. C. GILLILAND and RUSSELL FRASER

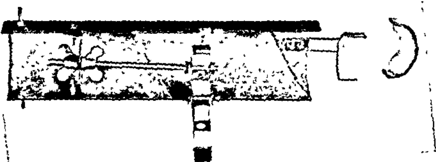
WE are engaged in developing a method of assaying thyrotrophic hormone by ^{131}I discharge and have been persuaded by this conference to a somewhat premature discussion on the results.

This method was chosen for several reasons. Cope (1938) showed that the chick was a very suitable animal, being readily available, sensitive to TSH, and having a thyroid gland of uniform histological nature. The discharge of ^{131}I as an index of thyrotrophic hormone activity was chosen as a result of the work of Keating *et al.* (1945), who showed that following the administration of TSH the discharge of iodine starts quickly, whereas the uptake of iodine is not apparent until after 24 hours. This discharge effect is the basis of one of the most sensitive assays so far put forward (De Robertis and Del Conte, 1944)

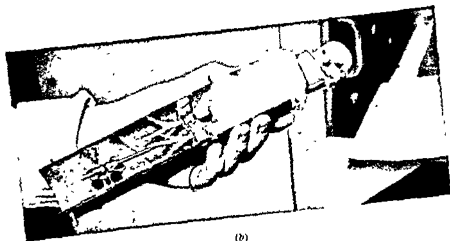
Perry (1951) showed that in the rat it is possible to prevent discharge of ^{131}I from the thyroid gland by the administration of sufficient thyroxine. Presumably this thyroxine acts upon the animal's pituitary, preventing it from forming its own thyrotrophic hormone, yet should not interfere with the effect of thyrotrophic hormone from external sources.

Method

Day-old Rhode Island Red × Light Sussex cross-bred cockerels were used. ^{131}I was administered before the chicks were fed and thereafter they were fed on a standard mash containing 0·30 μg. of iodine per g. Each chick was ringed with a number and a colour and subsequently reared in a standard brooder.



(a)



(b)

Fig. 1 Apparatus used to hold chick in counter.

Counter

The radioactivity in the thyroid gland of the chick was measured *in vivo*. The apparatus will be described in detail elsewhere (Arnott and Fossey, unpublished). The counter consists essentially of four Geiger-Muller tubes arranged in a ring and connected in parallel. They are so screened with lead that only a narrow area is radiosensitive, and, because of the arrangement of the counting tubes, slight lateral movements in that area do not significantly alter the rate of count.

The chick is so placed in the counter that its thyroid gland is in the radiosensitive region whilst the rest of its body, with any radioactivity that it may contain, is shielded from the counting system. By passing a fixed source of radioactivity through the radiosensitive zone it can be shown that there is a zone of maximum radiosensitivity about 2 cm. wide over which the rate of count is at its highest and does not alter appreciably. The rate of count outside the radioactive zone is not more than 10 per cent of the rate of count inside the zone. Thus the apparatus substantially measures the radioactivity of the thyroid gland. Excised thyroids gave approximately 104–106 per cent of the count observed *in vivo*.

The holder for the chick is shown in Fig. 1. The unanæsthetized chick rests comfortably in this. The head-piece is of Perspex to minimize interference The leg clamps are adjustable to allow for growth of the chick. On the outside at the end are two stops to ensure that the apparatus comes to rest at the same place each time, bringing the chick's thyroid into the radiosensitive area. As the chick is quite comfortable, indeed often goes to sleep in the apparatus, as many measurements as needed can be made

The test is in two stages The first stage consists of preparing the chick with an injection of thyroxine to hold the ^{131}I in the thyroid The second stage consists of administering TSH to some of these chicks and comparing the difference in discharge between the group of animals receiving TSH and a control group The difference is a measure of the dose of TSH administered.

^{131}I

Ten μc. carrier-free ^{131}I are administered, and approximately 10 per cent of the dose is in the thyroid glands at the end of the 24 hours, giving an average count of 1434/minute. It is believed it will have no radiation effect on the thyroid gland's ability to discharge iodine in the time of this experiment (Skanse, 1948).

Thyroxine

L-Thyroxine sodium is used, prepared freshly each day. It is administered at the time of the ^{131}I, although we are proceeding to determine the optimum time of administration. 30 μg. are administered intraperitoneally daily for at least three days, by which time retention is approximately complete (Fig. 2). We are still investigating the dose required to produce 100 per cent retention. Chicks' pituitaries, examined when the retentive effect is approximately complete, show an increase in the size and number of the eosinophils

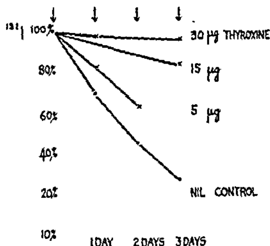
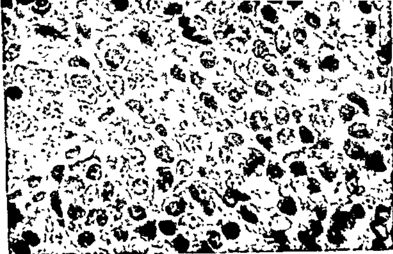
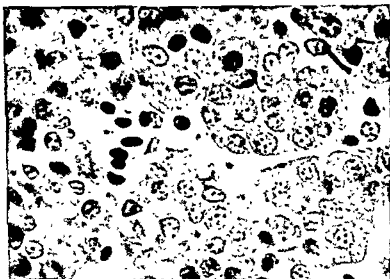


FIG. 2. Effect of varying daily doses of thyroxine on ^{131}I discharge



(*a*)



(*b*)

FIG. 3. Pituitaries of (*a*) control chick, (*b*) chick treated with 30 µg thyroxine daily for three days. (Pearse's modification of the periodic acid stain of Schiff, 525)

[*To face page 22*

^{131}I

Ten µc. carrier-free ^{131}I are administered, and approximately 10 per cent of the dose is in the thyroid glands at the end of the 24 hours, giving an average count of 1434/minute. It is believed it will have no radiation effect on the thyroid gland's ability to discharge iodine in the time of this experiment (Skanse, 1948).

Thyroxine

L-Thyroxine sodium is used, prepared freshly each day. It is administered at the time of the ^{131}I, although we are pro-

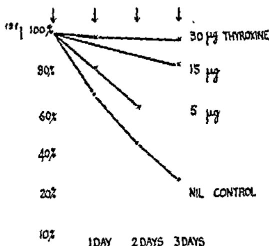
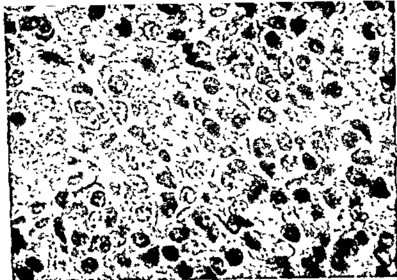
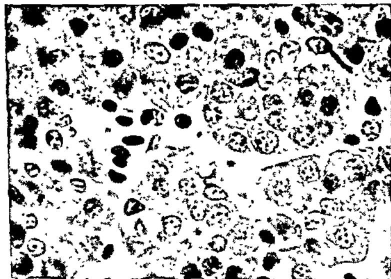


FIG 2. Effect of varying daily doses of thyroxine on ^{131}I discharge.

ceeding to determine the optimum time of administration. 30 µg. are administered intraperitoneally daily for at least three days, by which time retention is approximately complete (Fig. 2). We are still investigating the dose required to produce 100 per cent retention. Chicks' pituitaries, examined when the retentive effect is approximately complete,



(*a*)



(*b*)

FIG 3 Pituitaries of (*a*) control chick, (*b*) chick treated with 30 μg thyroxine daily for three days (Pearse's modification of the periodic acid stain of Schiff, × 325).

fluids. We are also trying to see if we can modify the sensitivity by altering the relationship between the thyroxine dose and the loading, to find what latitude there is before the sensitivity decreases. We are also experimenting with other methods of administration of continuous thyroxine, and with the use of goitrogens with thyroid to increase the sensitivity of the gland to TSH (Albert, Rawson, Riddell, Merrill and Lennon, 1947).

We would like to thank Miss J. Strudwick of the Biochemical Department, Mr. Arnott and Mr Fossey for their help with counting, and Mr. Wilmott of the Photographic Department.

REFERENCES

ALBERT, A., RAWSON, R. W., RIDDELL, C., MERRILL, P., and LENNON, B. (1947) *Endocrinology*, 40, 361.
ARNOTT, D. G., and FOSSEY, P *To be published.*
COPE, C. L. (1938). *J Physiol.*, 94, 358.
DE ROBERTIS, E., and DEL CONTE, E. (1944). *Rev. Soc. argent Biol.*, 20, 88.
KEATING, F. R, RAWSON, R. W., PEACOCK, W., and EVANS, R D. (1945). *Endocrinology*, 36, 137.
LARSON, R. A, KEATING, F. R., PEACOCK, W., and RAWSON, R. W. (1945) *Endocrinology*, 36, 160.
PERRY, W. F (1951). *Endocrinology*, 48, 643.
SKANSE, B N. (1948) *J. clin. Endocrinol.*, 8, 707.

CHICK ASSAY OF THYROTROPHIC HORMONE USING ^{32}P

A. C. CROOKE and JOYCE D. MATTHEWS

MANY different methods have been employed for the assay of thyrotrophin (TSH) with varying degrees of success. The chick was chosen because it has been shown, both by gravimetric and histological techniques, to be very sensitive to TSH. Radioactive phosphorus (^{32}P) was used as a measure of thyroid activity because it is a sensitive method of measuring changes in cellular metabolism.

Method

Ten two-day old male chicks were injected with a dose of Armour's TSH contained in a volume of 1 ml. of saline; later they received a dose of ^{32}P in 0·5 ml. of distilled water. The chicks were killed after a suitable time interval and the thyroids were dissected out rapidly and placed on weighed cover-slips which were weighed again. The thyroids on the cover-slips were placed on a platform in a lead castle at a fixed geometry to an end window Geiger counter, and counted. The control group of chicks which received ^{32}P only were all rated as registering 100 impulses per milligram of thyroid tissue and the TSH-treated groups were correlated to these

Results

It was found that an intraperitoneal injection of 10 μc. of ^{32}P contained in 0·5 ml. of distilled water was uniformly distributed after 2 hours. This dose was used and the chicks killed 2 hours afterwards in all subsequent work.

Sensitivity of the Breed. Three different strains of chicks were given 8 μg. of TSH followed by ^{32}P and were killed 4 hours after the first injection. It was found that first cross

Black Leghorn × Rhode Island Red, and pure bred Rhode Island Red chicks gave similar results, but that first cross Golden Legbar × Rhode Island Red chicks were unresponsive at this dose level. The BL/RIR were most consistently available and so were used for the rest of the experiments

Age of Chicks Groups of chicks aged 2, 4 and 6 days old were all injected with the same dose of TSH followed by ^{32}P, as in the previous experiment, and killed 4 hours after the first injection. There was no significant difference in responsiveness. Two-day-old chicks were used for all further investigations.

Site of Injection. Groups of chicks were injected subcutaneously, intraperitoneally and intramuscularly with the same dose of TSH and ^{32}P as before. Similar results were given by those receiving intraperitoneal and subcutaneous injections, but there was greater variation of results within the group which had the intramuscular injections. Intraperitoneal injections were used subsequently.

Number of Injections. A total dose of 8 μg. of TSH was given as a single dose to one group of chicks, and as two injections over 12 hours in another group, and a third group received six injections over 3 days. At this dose level only those having a single injection showed any response.

Time/Response Relationship. Different groups of chicks were given 8 μg. of TSH and left for different times, between 2 and 24 hours, before death. They received ^{32}P 2 hours before killing It was found that the maximum response occurred in those that had been given TSH for 4 to 6 hours. Subsequently there was a gradual reduction in response until 10 hours had elapsed, after which it remained constant (Fig. 1).

Dose/Response Relationship. Groups of 20 chicks were injected with 2, 4, 8, 12, 16 and 24 μg. TSH followed by ^{32}P, and killed 6 hours after the first injection. It was found that a 12-fold increase in dosage caused a $2\frac{1}{4}$-fold increase in response and that the log dose response could be represented by a straight line between the 2 and 12 μg. dose levels (Fig. 2).

Discussion

The chick assay using ^{32}P has several advantages. The animals are readily available and cheap because the light breed males are normally destroyed at the hatcheries. They are killed within 24 hours, which reduces the problem of caging and feeding them. The method is simple because each chick requires only two injections. It is very rapid and

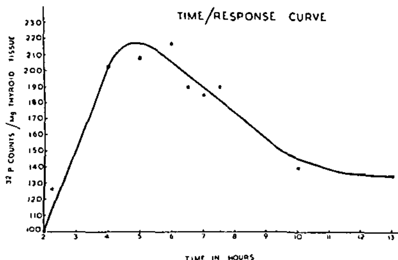


FIG. 1. Time/response curve showing the uptake of radioactive phosphorus by the chick thyroid at different intervals of time after the injection of a dose of 8 μg of thyrotrophic hormone.

can be completed in one day. It is not possible to compare the method accurately with others because there is no international standard, but it appears to be very sensitive. The two preparations from Armour and Company which have been used were said to contain between 9 and 12·5 Junkman Schoeller Units (J.S.u.)/mg Each could be measured at a dose of 2 μg. per chick, indicating that the chick is sensitive down to about 0 02 J S.u. This is of the same order of sensitivity as that claimed by Dvoskin (1947) using a histological method in the chick, but we have been unable to

repeat his results. Other workers have claimed a greater sensitivity than this. D'Angelo, Paschkis, Gordon and Cantarow (1951) and De Robertis (1948) have devised very elaborate methods which they claimed to be sensitive down to a level of 0·0002 J.S.u. Both are open to the same criticism. In De Robertis' method a 5,000-fold increase in dose

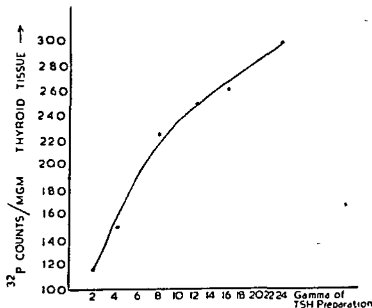


FIG 2 Dose/response curve showing the uptake of radioactive phosphorus by the chick thyroid six hours after the injection of varying amounts of thyrotrophic hormone

was required to produce a 5-fold increase in response. In the method of D'Angelo *et al.* a 500-fold increase in dose was required to produce a 2-fold increase in thyroid cell height and in hind limb growth, the response depending on the difference between these two variables. Their dose/response curves are so flat that it is questionable whether they are of practical use until they reach a dose of the same order as that used in our present method.

Finally, the method appears to be specific. A group of chicks was injected with 0·6 i.u. ACTH and it produced no response. Doses of 0·9 i.u. had proved fatal in earlier experiments. Another group was injected with 5 i.u. of gonadotrophin but it also gave no response.

The method has been applied to urine from a patient with exophthalmic ophthalmoplegia and has given a measurable response with the ultrafiltrate from 15 ml. per chick on two occasions. Further work on the isolation of thyrotrophic hormone from urine is in progress.

Acknowledgments

We wish to express our gratitude to Mr. D. G Bodmeade, Manager of Sterling Poultry Products Ltd., Worcester, for the generous gift of chicks, and to Dr. S. L Steelman of Armour and Company, Chicago, for the supplies of thyrotrophic hormone

REFERENCES

D'ANGELO, S A, PASCHKIS, K. E, GORDON, A. S, and CANTAROW, A (1951) *J. clin. Endocrinol.*, 11, 1237

DE ROBERTIS, E. (1948). *J. clin. Endocrinol.*, 8, 956.

DVOSKIN, S. (1947) *Endocrinology*, 41, 220.

DISCUSSION

GADDUM. Is there any evidence about the comparative sensitivity of the three methods?

FRASER Our lowest dose which was effective was 0 025 Armour units.

CROOKE: Ours was 0 02

COPE: The point I wish to make is possibly important for the relative clinical values of these methods. The method of Fraser and Gilliland involved the preliminary saturation of the chick with thyroxine, and the others didn't The difficulty with assay in blood is that the total activity is a balance between the thyroid hormone which inhibits, and the thyrotrophic hormone which stimulates thyroid tissue This is not likely to be a serious difficulty with the Gilliland and Fraser method, as the chick is already saturated with thyroxine and any effect will be due to TSH alone.

GADDUM. Dr. Crooke, could you saturate your chicks with thyroxine?

CROOKE: Yes, we could, but we haven't tried yet

FRASER: Dr. Mason said that ^{32}P was concerned with cell division Isn't it just as likely that it is concerned with this discharge we are measuring? Presumably the assay by ^{32}P would still work after loading with thyroxine.

MASON: We have no data, but it is a possibility to be explored

repeat his results. Other workers have claimed a greater sensitivity than this. D'Angelo, Paschkis, Gordon and Cantarow (1951) and De Robertis (1948) have devised very elaborate methods which they claimed to be sensitive down to a level of 0·0002 J.S.u. Both are open to the same criticism. In De Robertis' method a 5,000-fold increase in dose

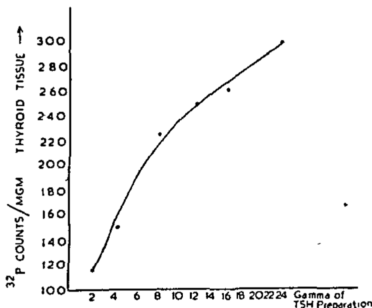


FIG 2. Dose/response curve showing the uptake of radioactive phosphorus by the chick thyroid six hours after the injection of varying amounts of thyrotrophic hormone.

was required to produce a 5-fold increase in response. In the method of D'Angelo *et al.* a 500-fold increase in dose was required to produce a 2-fold increase in thyroid cell height and in hind limb growth, the response depending on the difference between these two variables. Their dose/response curves are so flat that it is questionable whether they are of practical use until they reach a dose of the same order as that used in our present method

repeat his results. Other workers have claimed a greater sensitivity than this. D'Angelo, Paschkis, Gordon and Cantarow (1951) and De Robertis (1948) have devised very elaborate methods which they claimed to be sensitive down to a level of 0·0002 J.S.u. Both are open to the same criticism. In De Robertis' method a 5,000-fold increase in dose

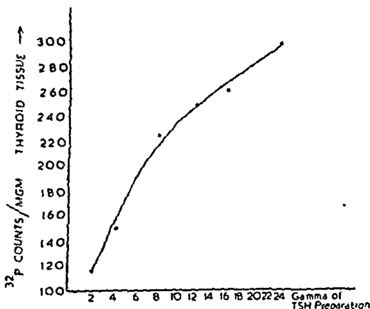


FIG. 2. Dose/response curve showing the uptake of radioactive phosphorus by the chick thyroid six hours after the injection of varying amounts of thyrotrophic hormone.

was required to produce a 5-fold increase in response. In the method of D'Angelo *et al.* a 500-fold increase in dose was required to produce a 2-fold increase in thyroid cell height and in hind limb growth, the response depending on the difference between these two variables. Their dose/response curves are so flat that it is questionable whether they are of practical use until they reach a dose of the same order as that used in our present method.

Crooke. It's only in the last fortnight that we've tried extracting ... to say anything ... but I wouldn't like to

... out, of course

Butt We have tried benzoic acid, and we obtained rather toxic extracts

Cope: You would if the urine was old It's not toxic with fresh urine.

Loraine Dr. Rawson has tried to repeat De Robertis' method and has been unable to do so. He said that its chief difficulty was its lack of specificity· he obtained similar results from injecting saline as from injecting so-called active preparations

Gaddum But the tadpole hasn't had a chance yet to show its possibilities

Loraine· I don't think so. I don't think anybody apart from Dr D'Angelo is doing that assay.

Astwood: Have any of these investigators noted whether corticotrophin given with the thyrotrophin would interfere with the thyrotrophic response?

C. J O. R. Morris The ACTH content of this preparation 317–51 is very low, according to Armour. So we have in effect been injecting some ACTH with thyrotrophin in that case That is certainly true of our preparation CA, which also contains ACTH

Dedman: ACTH alone has no effect on ^{32}P uptake by the thyroid.

Gaddum: Have you tried the effect of adding ACTH to your thyrotrophin?

Mason· We have tried adding propylthiouracil to increase the sensitivity. Our data on that are very scanty, but it does appear to increase *the sensitivity*

Gaddum Do you know at all?

G[illegible]: I thought some of your lines weren't quite straight. It

between responses befo
gave straight lines. I
your results

DEDMAN: We've done some statistics on these lines and also ratio
[illegible] and it seems to be all right for the centre of
onse, and if you take the
ld increase in the dose over
paration which Dr. Mason
'm used to computing them
—not s/b

MACLAGAN: I would like to ask about the technique of the counting. I noticed that in the two methods in which the thyroids are dissected
[illegible] rather

do it, and I am not sure that one gets much [illegible] the result as percentage of the dose given. One is always concerned with what the control values are, and it doesn't seem to me to matter what units you use, provided they are standard for your experimental conditions.

CROOKE: I think that's a very important point in connection with ³²P because of its soft emission. The advantage of using the chick
[illegible] that the amount of radiation absorbed
thyroids from the ten
coverslip.
ld you get a reasonable

nore complicated.
up with those of
ysectomized rat?
P work.
the same animal?
very rough guess,
't go further than
see how you can
get any comparison.

C. J. O. R. MORRIS: Sayers assayed some 317-51 by his ¹³¹I method and found about the same sensitivity.

[illegible] thods of extraction
ltra-

CROOKE: It's only in the last fortnight that we've tried extracting urine and I think probably it's a little too soon to say anything

BUTT: We have tried your kaolin technique, but I wouldn't like to say that we have extracted thyrotrophin.

COPE: Might I suggest that in this extraction you should not forget the benzoic acid method, originally suggested to me by Harington (Cope, *Quart. J. Med.*, 1938, 7, 151). It seemed to recover quantitatively thyrotrophin added to urine. That doesn't prove it gets the natural substance out, of course

BUTT: We have tried benzoic acid, and we obtained rather toxic extracts.

COPE: You would if the urine was old. It's not toxic with fresh urine.

ASTWOOD. Dr. Trikojus, who has recently been with us, has found that sometimes the benzoic acid method does not work. What the variable is he wasn't able to determine.

GADDUM. There's probably not much chance of any of these methods working when applied to blood Has anyone any experience of De Robertis' method using tadpoles?

LORAINE Dr. Rawson has tried to repeat De Robertis' method and has been unable to do so. He said that its chief difficulty was its lack of specificity he obtained similar results from injecting saline as from injecting so-called active preparations.

GADDUM: But the tadpole hasn't had a chance yet to show its possibilities.

LORAINE I don't think so I don't think anybody apart from Dr. D'Angelo is doing that assay.

ASTWOOD Have any of these investigators noted whether corticotrophin given with the thyrotrophin would interfere with the thyrotrophic response?

C. J. O R MORRIS: The ACTH content of this preparation 317–51 is very low, according to Armour So we have in effect been injecting some ACTH with thyrotrophin in that case That is certainly true of our preparation CA, which also contains ACTH.

DEDMAN ACTH alone has no effect on ^{32}P uptake by the thyroid

GADDUM: Have you tried the effect of adding ACTH to your thyrotrophin?

MASON We have tried adding propylthiouracil to increase the sensitivity Our data on that are very scanty, but it does appear to increase the sensitivity.

ASTWOOD: Even after hypophysectomy?

MASON: No, I was merely thinking of D'Angelo's work on the clinical side, when he reported several high levels of TSH in patients with acromegaly.

GADDUM: Have you any idea what is the maximum amount of growth hormone or ACTH which should be allowed in standard preparations of TSH?

LI: They should be completely free if possible.

GADDUM: ACTH isn't so easy to get rid of.

LI: I think it can be more easily removed than GH, since the major

[illegible] ted the

MASON: It is on our programme.

Part III—Gonadotrophins and Prolactin

EFFECTS OF CHORIONIC AND EQUINE GONADOTROPHINS ON HYPOPHYSECTOMIZED IMMATURE RATS

R. MORICARD

THE standardization of gonadotrophins sets a difficult technical problem. We have investigated here the effects induced by chorionic and equine gonadotrophins on immature female hypophysectomized rats.

At the third conference on hormone standardization which took place at Geneva in 1938 under the auspices of the Hygiene Organization of the League of Nations, Evans (1939) was the only one to suggest the use of the immature hypophysectomized female rat. He took as the criterion of gonadotrophic activity the increase in weight of the ovary.

All the other members of the commission for standardization used for their biological trials immature animals which were not hypophysectomized. Usually vaginal keratinization and ovarian luteinization were taken as criteria, according to the technique of B. Zondek (1926). Two standards were established one for the chorionic gonadotrophin discovered by Aschheim (1926) in the urine of pregnant women; and the other for the equine serum gonadotrophin discovered by Cole and Hart (1930) in the serum of pregnant mares.

Since 1929 the work of Evans and Simpson has shown that gonadotrophic extracts of the anterior pituitary have a different physiological action from chorionic gonadotrophins.

The Effects of Hypophysectomy in the Rat, using P. E. Smith's Technique

The rats that we have used are a little lighter than the Wistar rat. They were fed on "Ratigen" (a powder, comparable to tablets of Purina Chow), without added milk

In these rats the vaginal opening usually appears when they weigh about 60 g., and the first keratinization when they weigh about 90 g. In the immature rats weighing 50 g. the ovary weighs about 5 mg.; there are already follicles with cavities, but no corpora lutea.

It is customary to consider that P. E. Smith's hypophysectomy is a total hypophysectomy, and it is often forgotten that Smith (1930) stated on p. 210, footnote 2, of his paper: "In my operations upon the pituitary the pars tuberalis has in no case been removed, 'Hypophysectomy' meaning, in accordance with common usage, the removal of the anterior and the posterior (intermediate and neural) lobes. The pituitary stalk is also left intact." The pars *tuberalis* is embryologically an expansion of the pars anterior, and in the *strict sense*, the "hypophysectomy" of the rat is systematically incomplete.

In carrying out P. E Smith's "hypophysectomy", we have preferred the sub-thyroid route suggested by H. Selye. Since 1945 we have carried out "hypophysectomies" on 109 adult female rats and 77 immature rats, to investigate the effect on ovogenesis and meiosis (Moricard, 1948; Moricard and Gothie, 1949). We think that studying the base of the skull with a binocular loupe is inadequate for determining the absence of anterior hypophyseal remnants. If there is a small remnant in the trephine opening or in the neighbourhood of the carotids, it is not detected in the examination of the base of the skull. To detect hypophyseal remnants a series of sections from the base of the skull are necessary. We have established that frontal sections are preferable to sagittal sections. The base of the brain and the sphenoid are lifted up *en bloc* and the trigeminal nerve cut. The block is stained *in toto* with carmine, and is cut and mounted as a complete series. It is at the level of the vascular hypophyseal carotid pedicle that the anterior hypophyseal remnants are most often seen.

Effects of Gonadotrophins on the Hypophysectomized Adult Female Rat

It is well known that in the adult rat of about 100 g. which has been hypophysectomized without an anterior hypophyseal residue, there is no vaginal keratinization, the weight of the ovary remains around 12 mg., and histological examination shows the persistence of the corpus luteum, as Smith established. If there is an anterior pituitary residue, the corpus luteum regresses and the ovarian weight may be less than 12 mg. If the residue is greater than 10 per cent of the weight of the pituitary, some follicles enlarge, new corpora lutea are formed, and the ovarian weight can be greater than 12 mg. Owing to these variations the ovary of the adult rat is poor material for recognizing easily the effects of hypophysectomy. If complete hypophysectomies have been carried out successfully (verified by studying the base of the skull) it is possible to induce vaginal keratinization in the adult hypophysectomized rat with doses of 10–50 I.U. of chorionic gonadotrophin or equine gonadotrophin, injected over 6 days, the animals being killed 144 hours after the beginning of the treatment

The Effect of Hypophysectomy in the Immature Rat

Some effects of hypophysectomy on the ovary of the immature rat have been studied by H. Selye. In 1949, during one of the author's visits to Columbia University, P. E. Smith drew attention to the fact that he had not studied the effects of hypophysectomy on the ovary of the immature female rat.

(*a*) *Stabilization of Body Weight.* The operation is done when the animals weigh approximately 50 g. If, after operation, the weight of the animal increases by 20 g. or more, the hypophysectomy is certainly incomplete; this has been observed with residues consisting of from 2–7 per cent of the anterior pituitary. If the weight increases by less than 10 g., or if it is stable, or even if it decreases, complete absence of an anterior pituitary residue is very probable,

In these rats the vaginal opening usually appears when they weigh about 60 g., and the first keratinization when they weigh about 90 g. In the immature rats weighing 50 g. the ovary weighs about 5 mg.; there are already follicles with cavities, but no corpora lutea.

It is customary to consider that P. E. Smith's hypophysectomy is a total hypophysectomy, and it is often forgotten that Smith (1930) stated on p. 210, footnote 2, of his paper: "In my operations upon the pituitary the pars tuberalis has in no case been removed, 'Hypophysectomy' meaning, in accordance with common usage, the removal of the anterior and the posterior (intermediate and neural) lobes. The pituitary stalk is also left intact." The pars tuberalis is embryologically an expansion of the pars anterior, and in the strict sense, the "hypophysectomy" of the rat is systematically incomplete.

In carrying out [illegible] re have preferred the sub- [illegible] Since 1945 we have car [illegible] adult female rats and 77 immature rats, to investigate the effect on ovogenesis and meiosis (Moricard, 1948; Moricard and Gothie, 1949). We think that studying the base of the skull with a binocular loupe is inadequate for determining the absence of anterior hypophyseal remnants. If there is a small remnant in the trephine opening or in the neighbourhood of the carotids, it is not detected in the examination of the base of the skull. To detect hypophyseal remnants a series of sections from the base of the skull are necessary. We have established that frontal sections are preferable to sagittal sections. The base of the brain and the sphenoid are lifted up *en bloc* and the trigeminal nerve cut. The block is stained *in toto* with carmine, and is cut and mounted as a complete series. It is at the level of the vascular hypophyseal carotid pedicle that the anterior hypophyseal remnants are most often seen.

Effects of Gonadotrophins on the Hypophysectomized Adult Female Rat

It is well known that in the adult rat of about 100 g. which has been hypophysectomized without an anterior hypophyseal residue, there is no vaginal keratinization, the weight of the ovary remains around 12 mg., and histological examination shows the persistence of the corpus luteum, as Smith established. If there is an anterior pituitary residue, the corpus luteum regresses and the ovarian weight may be less than 12 mg. If the residue is greater than 10 per cent of the weight of the pituitary, some follicles enlarge, new corpora lutea are formed, and the ovarian weight can be greater than 12 mg. Owing to these variations the ovary of the adult rat is poor material for recognizing easily the effects of hypophysectomy. If complete hypophysectomies have been carried out successfully (verified by studying the base of the skull) it is possible to induce vaginal keratinization in the adult hypophysectomized rat with doses of 10–50 I.U. of chorionic gonadotrophin or equine gonadotrophin, injected over 6 days, the animals being killed 144 hours after the beginning of the treatment

The Effect of Hypophysectomy in the Immature Rat

Some effects of hypophysectomy on the ovary of the immature rat have been studied by H. Selye. In 1949, during one of the author's visits to Columbia University, P. E. Smith drew attention to the fact that he had not studied the effects of hypophysectomy on the ovary of the immature female rat

(a) *Stabilization of Body Weight.* The operation is done when the animals weigh approximately 50 g. If, after operation, the weight of the animal increases by 20 g or more, the hypophysectomy is certainly incomplete, this has been observed with residues consisting of from 2–7 per cent of the anterior pituitary. If the weight increases by less than 10 g, or if it is stable, or even if it decreases, complete absence of an anterior pituitary residue is very probable,

but is not certain—anterior pituitary residues are sometimes poorly vascular or lightly coloured, and in these conditions the weight of the animal becomes stable, or even regresses, as if the removal of the anterior pituitary were complete.

(*b*) *Regression in Weight of the Ovary*. At the time of hypophysectomy the ovarian weight is about 5 mg.; 15 days after hypophysectomy the ovarian weight is in the neighbourhood of 2 mg.; and 30 days after hypophysectomy the ovarian weight is about 1 mg. (see Table I). The variations in weight are quite important. If one considers the weight of the ovary in an animal of 50 g., it is apparent that the following ratio, $\frac{Ov_1 \times 50}{W_0}$ (where Ov_1=ovarian weight on the day of hypophysectomy; and W_0=the weight of the rat at the time of removal of the ovary) increases when the weight of the rat is less than 50 and decreases when the weight is more than 50. With animals which are too light or too heavy, this ratio is debatable. In our experience, the above ratio is between 0·6 and 1·8 in immature rats in which the anterior pituitary residue is below 5 per cent of the weight of the pituitary. Thirty days after hypophysectomy, ovarian weight is relatively stable, and the ratio is in the neighbourhood of 1. Histologically, there is no formation of cavities in the follicles, whose diameter is usually under 280 μ.

Effects of Chorionic Gonadotrophin on the Hypophysectomized Immature Female Rat

Of the 12 rats used, four had only a partial hypophysectomy: in a rat which had gained 22 g. and one which had gained 17 g., in frontal section there was an anterior pituitary remnant which had a maximum surface of 0·1 mm.^{2}; in animal No. 1052, which gained only 2 g., a remnant of 0·08 mm.^{2} was found; and in animal No. 1093, which had gained 5 g., a residue of 0·02 mm.^{2} (Table I).

In these 12 "hypophysectomized" animals, from 1 to 500 i.u. chorionic gonadotrophin were injected, administered in

Table I

EFFECT OF CHORIONIC GONADOTROPHIN ON HYPOPHYSECTOMIZED IMMATURE FEMALE RATS

No	Days	W_0 g	W_1-W_0 g	Ant pit remnant mm³	V_1	Ov_1 mg	$Ov_1 \times 50$ / W_0	Foll diam μ	Int	Dosage i u (No of inj)	V_2	Ov_2 mg	Foll diam μ	CL	Int	Uterus mg
1060	37	57	− 5	0	VF	1 7	1 5	180	0	500 (6)	VF	5	315	0	++++	32
1094	28	37	+ 5	0	VF	1	1 3	180	0	500 (6)	VF	3	300	0	++++	22
1095	28	34	+22	0 1	VF	1 3	1 9	220	0	500 (6)	VF	4 1	350	0	++++	25
989	39	50	0	0	VF	1	1	250	0	50 (4)	VF	3 5	130	0	+++	39
1004	37	45	+ 8	0	VF	1	1 1	230	0	50 (4)	VF	4	215	0	+++	20
1052	30	63	+ 2	0 08	VF	1 7	1 3	300	0	50 (6)	VF	6	200	0	+++	34
1093	28	48	+ 5	0 02	VF	1 4	1 5	225	0	50 (6)	VF	3 5	150	0	+++	40
1080	6	33	+ 2	0	VF	1 3	2	250	0	50 (6)	VF	3 2	230	0	+++	25
984	42	50	− 3	0	VF	1	1	215	0	2 (4)	VF	1 0	180	0	++	20
1003	37	45	+ 4	0	VF	1 5	1 6	280	0	2 (4)	VF	1 9	250	0	++	27
990	38	50	− 2	?	VF	1	1	215	0	1 (4)	VF	1 5	200	0	0	34
1002	37	45	+17	0 1	VF	1	1 1	230	0	1 (4)	VF	1 6	170	0	0	28
						1 24	1 35	231								

Days—number of days since hypophysectomy.
W_1—the weight of the rat on the day of hypophysectomy.
W_0—the weight of the rat on the day when the first ovary (Ov_1) is removed.
V_1—the state of the vagina on the day of hypophysectomy. VF—closed vagina.
Ov_1—the weight (in mg) of the ovary on the day of hypophysectomy.

Dosage—Gonadotrophin administered as one 5 cc. injection per day. Total number of injections in parentheses.
V_2—is the state of the vagina after treatment.
Ov_2—the weight (in mg.) of the ovary after treatment.
CL—formation of corpus luteum.
Int—reaction of the interstitial tissue (enlargement of the cells).
Uterus—the weight of the uterus (in mg.) after treatment. (The weight of the uterus after hypophysectomy is around 30 mg.)

4 or 6 injections. The animals were *killed 24 hours* after the last injection. At the beginning of the experiment all the animals had closed vaginæ. It was found that hypophysectomized animals with an anterior pituitary remnant whose maximum surface in frontal section was 0·1 mm.[2] or less, behaved as if the anterior hypophysectomy were complete.

With doses of 1 to 50 international units* there is no increase in follicular diameter: the mean follicular diameter before treatment being 225 μ, and after treatment 150 μ. After treatment with 50 i.u. chorionic gonadotrophin, ovarian weight can increase as much as four times.

With doses of 500 i.u. in three animals there is an increase of about 100 μ in follicular diameter, with no cavity formation *in the follicles* and no formation of a corpus *luteum*.

A constant result with doses of 2 to 500 i.u. is an increase in size of the interstitial cells. There is hypertrophy of these cells without hyperplasia. There is no vaginal keratinization and no increase in uterine weight, which remains at approximately 27 mg. There is, therefore, no secretion of œstrogen by interstitial ovarian tissue hypertrophied under the influence of chorionic gonadotrophin.

Effects of Equine Gonadotrophin on the Ovary of the Hypophysectomized Immature Female Rat

Of 13 hypophysectomized rats, four had anterior hypophyseal residues, and of these, two had gained weight and *two lost (Table II).*

The results obtained by treatment with equine gonadotrophin are related to the dose, to the duration of treatment (96 or 144 hours), to the presence or absence of an anterior pituitary remnant (even a very small one) and to the time after hypophysectomy. From 2 to 50 international units† were injected.

With 2 or 5 i.u. the increase in weight of the ovary is very

*This experiment was carried out with the original international standard

†*A part of this experiment was carried out with the original international* standard.

Table II

EFFECT OF EQUINE GONADOTROPHIN ON HYPOPHYSECTOMIZED IMMATURE FEMALE RATS

No	Days	g W_0	g W_1-W_0	mm³ Ant pit residue	V_1	mg Ov_1	$\frac{Ov_1 \times 50}{W_0}$	μ Foll diam	Int	Dosage i u (No of inj)	V_2 M	P	E	K	mg Ov_2	μ Foll diam	CL	Int	Uterus
1000	72	68	−11	0	VF	0 8	0 6	170	0	50 (6)				VF	3 5	50	0	+++	30 5
1053	30	56	− 1	0	VF	1	0 9	200	0	50 (6)	+	+	++		11 9	500A	+	+++	215
1107	17	38	− 2	0 04	VF	1 3	1 7	215	0	50 (6)				++++	23	660A	+	+++	122
1120	15	38	− 2	?	VF	1 4	1 7	340	0	50 (6)				++++	10 5	660A	0	+++	365
1115	6	45	− 3	0	VF	1 3	1 5	250	0	50 (6)				++++	18	690A	++	+++	442
998	38	50	+ 1	?	VF	1	1	230	0	50 (4)				VF	6 5	540A	0	+++	65
1009	36	80	+12	0 4	VF	2 5	1 6	215	0	50 (4)				VF	13	540A	0	+++	98
1081	6	37	+ 1	0	VF	1 7	2 3	230	0	50 (4)		+	++		13 5	580A	+	+++	141
1114	6	50	− 4	0	VF	2	2	230	0	50 (4)				++++	24	530A	++	+++	422
1067	37	58	+ 2	0	VF	1 2	1	200	0	25 (6)				VF	5 8	50	0	+++	20 5
1068	37	52	− 2	0	VF	1	1	250	0	25 (4)				VF	8	580A	0	+++	132
995	38	50	+ 8	0 05	VF	1 5	1 5	250	0	5 (4)		++	+		3	200	0	+	31
1018	34	95	−17	0 002	VF	2 1	1 1	250	0	2 (4)				VF	2	215	0	0	41
						1 45	1 37	233											

For explanation see the table on chorionic gonadotrophin

M—mucus
P—leucocytes
E—epithelial cells
K—keratinized cells
Foll diam.—A—follicular cavity (antrum)

slight. With doses of 25 i.u., the vagina may remain closed, in which case the weight of the ovary is increased 4–8 times. There is a very marked interstitial response. If follicular cavities are formed, there is an increase in uterine weight, demonstrating the existence of an œstrogenic action; if there is no cavity formation, the uterine weight does not increase.

With doses of 50 i.u. in four injections, the animals are killed 96 hours after the first injection. This follows the technique which we used for standardization in the immature rat. If the hypophysectomy is recent (6 days), the vagina opens and keratinization of the vagina can be seen. On the other hand, if the hypophysectomy is old (36–38 days) the vagina remains closed. Uterine weight increases if there is follicle formation.

With doses of 50 i.u. in six injections, the animals are killed 144 hours after the first injection. In these animals equine gonadotrophin stimulates œstrogenic effects, secondary to follicle development and cavity formation. In rats hypophysectomized less than 30 days previously there can be formation of a corpus luteum. In a rat of 68 g., hypophysectomized 72 days previously, the weight of the ovary was 0·8 mg., and after treatment it was 5·5. There was interstitial hypertrophy, but no follicle development, and the uterus remained atrophic; it weighed 36·5 mg. This isolated result confirms the fact that œstrogen secretion does not necessarily accompany interstitial ovarian hypertrophy.

Discussion

It is well known that in the adult and immature hypophysectomized rat, pituitary extracts are able to stimulate follicle development, maturation of the ova, ovulation, and formation of a corpus luteum.

Gonadotrophic extracts from the urine of pregnant women (chorionic gonadotrophin) in the adult hypophysectomized rat stimulate œstrus and produce keratinization of the vagina, but not follicle formation. On the other hand, in the immature hypophysectomized rat there is hypertrophy of the

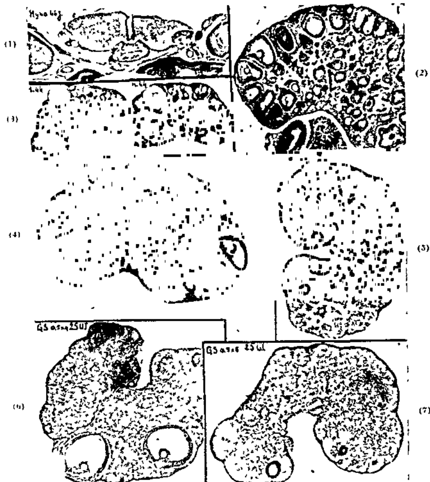


Fig. 1 Effects of gonadotrophins on the immature hypophysectomized rat

1 Base of skull after hypophysectomy Pars tuberalis not removed
2 Ovary of 50 g immature intact rat Presence of follicles with antrum No corpora lutea
3 I ... trophy
4 ... units) Follicular development,
5 ... hypertrophy No œstrus No
6 ... y Follicular development and
7 Serum gonadotrophin Interstitial hypertrophy No cavity formation No œstrus

[To face page 40

REFERENCES

Aschheim, S (1926). *Med. Klinik.*, 22, 2023.
Cole, H. H, and Hart, G. H. (1930). *Amer. J. Physiol.*, 93, 57.
Evans, H M. (1939). *Bull. org. Hyg.*, 8, No. 6, 952.
Moricard, R. (1940). *Bull. biol*, Suppl. 28, p. 279.
Moricard, R. (1948) *J. Physiol. Path. gén.*, 40, 273A.
Moricard, R, and Gothie, S. (1949). *J. Physiol. Path. gén.*, 41, 184A.
Smith, P. E. (1926) *Anat. Rec.*, 32, 221.
Smith, P E (1930). *Amer. J. Anat.*, 45, 205.
Zondek, B. (1926) *Geburtsh Gynäk.*, 90, 372.

DISCUSSION

Gaddum. How are these results related to work on the separation of the gonadotrophins in pituitary extracts?

R. Moricard Our extract was made from the hypophysis of the bull and contained both follicle stimulating and luteinizing factors, without separation

Robson Can these various changes, histological and in vaginal keratinization, be explained in terms of various amounts of two fractions?

R. Moricard· I don't think that it is the explanation, that there are two kinds of substance in the extract of pregnant urine or in equine gonadotrophin I feel that we should think in terms of the response of the ovary. In using hypophysectomized immature rats, you do not make the mistake of having corpora lutea in the ovary; and if you have no corpora lutea in the ovaries you have no œstrus The response also depends on the time after hypophysectomy and the amount of pituitary tissue remaining. It seems to me that it is better to try to understand the difference in the response of the rat by thinking about the target organ, the ovary, than by trying to think in terms of different pharmacodynamic activities of several substances.

Gaddum· Have you done any experiments in which the animals first received serum gonadotrophin in order to form follicles, and then chorionic gonadotrophin afterwards to see whether it would liberate œstrogens?

R. Moricard: Yes, we have tried experiments in five or six animals, but it is very complicated because the response of the immature ovary

follicle which is able to respond to the chorionic gonadotrophin.

Rowlands· Did you observe the occurrence of ovulation after the injection of pregnant mares' serum?

R. Moricard I am not sure. We made sections of the ovary and also of the tube. We have never seen ova in the tube, but we have obtained

some corpora lutea without oocytes. It is possible that in some cases we have induced ovulation, but it is not certain.

ROWLANDS. That confirms some experiments which I did in collaboration with P. C. Williams some years ago. Hypophysectomized rats (5 days or 7 days after the operation) were injected with pregnant mares' serum; we got a very high level of follicular growth but few if any of the follicles ovulated. By then injecting chorionic gonadotrophin we could liberate eggs at will.

F. MORICARD: How many days between the two injections?

ROWLANDS. The chorionic gonadotrophin was injected 4 or 5 days after the injection of pregnant mares' serum, i.e., about ten days after hypophysectomy.

R. MORICARD: Our results agree with this. As shown in our table, in animals hypophysectomized for 5 or 6 days growth of the follicles was very easily produced with equine gonadotrophin. But if the same equine gonadotrophin was injected into animals which had been hypophysectomized for more than a month, it was not possible to produce much growth of the follicles. If you study the biometric curve of the regression of the weight of the ovaries you can obtain a level of 1 mg. ovarian weight only after one month. For me an animal hypophysectomized for only 6 days is not a truly hypophysectomized animal.

LORAINE. Did you notice ovarian hyperæmia in any of your experiments?

R. MORICARD. Yes, there is a very great hyperæmia of the ovaries. The ovaries were very red.

LORAINE: That was with chorionic gonadotrophin?

R. MORICARD. Yes.

CHROMATOGRAPHY OF URINARY GONADOTROPHINS

W. R. BUTT and A. C. CROOKE

THE excretion of gonadotrophins by women with functional amenorrhœa during the reproductive period of life is sometimes within the normal range for menstruating women and sometimes it rises to the levels found in women past the menopause. When it is measured daily it may be observed to fluctuate in a manner resembling the normal periodic fluctuation during the menstrual cycle.

This study was undertaken to investigate whether any qualitative changes occur in the gonadotrophins excreted by women with amenorrhœa and with normal cycles. It was subsequently extended to women past the menopause and during pregnancy.

Since the mouse uterus assay does not differentiate qualitatively between follicle stimulating and luteinizing effects, the fractionation of urinary gonadotrophins was attempted by chemical means.

Methods

The gonadotrophins were extracted from urine by the kaolin adsorption method of Dekanski (1949) and then treated by the chromatographic technique described by Swingle and Tiselius (1951). Tricalcium phosphate was used as an adsorbent, and it was prepared and mixed with five parts by weight of hyflosupercel, as described by the authors. The size of column finally adopted was 6 cm. $\times$ 1·3 cm.

Sodium chloride solution and phosphate buffers were tried as eluting agents, but the striking changes in the rate of elution of protein for small changes in salt concentration of the eluting agent, reported by Swingle and Tiselius, were not repeated for gonadotrophins.

The solutions finally chosen were disodium hydrogen phosphate (Na_2HPO_4) (0·002 M) and trisodium phosphate (Na_3PO_4) (0·02 M). The crude gonadotrophin prepared by kaolin adsorption was reprecipitated from 80 per cent acetone and then dissolved in a small amount of Na_2HPO_4. The soluble material was applied to the top of the column and elution commenced with this solvent.

In most cases two coloured bands appeared, the first being eluted with the front, and the second remaining within 1 cm. of the top of the column. The first band was eluted completely in about 15 ml. Na_2HPO_4 This will be referred to as Gonadotrophin "A". The solvent was then changed to Na_3PO_4, which eluted the second band in a further 20 ml. It will be referred to as Gonadotrophin "B".

The columns were run at 20°C., and as quickly as possible, using about 3 cm. Hg pressure. They were completed in 7 to 8 hours.

The eluted material was then assayed immediately or precipitated with acetone and stored in a dry state until ready for assay.

The advantage of this particular system is that the mild adsorbents and aqueous media which are used are not likely to affect the activity of the protein hormones

The mouse uterus assay described by Levin and Tyndale (1936) was used and a 100 per cent increase in uterus weight was regarded as a positive response. For the assay of crude gonadotrophins two mice were injected at each of three dilutions. In some instances the rat prostate method of Loraine (1950) was used as a measure of luteinizing substance.

Results

The recovery of gonadotrophins from the phosphate columns was satisfactory. Crude gonadotrophin was assayed by the mouse uterus method and known amounts applied to columns. In four experiments the recovery of material in the eluates was complete, as far as bioassay could demonstrate.

The gonadotrophins excreted by a normally menstruating

woman were first investigated. Gonadotrophin "A" was present for one or two days early in the cycle and for part of the luteal phase. Gonadotrophin "B" was only recognized

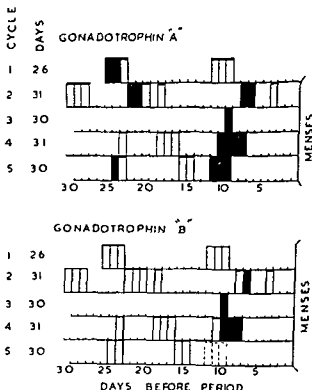


FIG. 1. Urinary excretion of gonadotrophins in five normal menstrual cycles.

Open columns Inactive by mouse uterus assay.
Black columns. Active by mouse uterus assay
Broken columns Inactive by rat prostate assay.

during a brief part of the luteal phase in three separate cycles (Fig. 1). No luteinizing hormone has been demonstrated so far by the rat prostate assay, but this is probably because insufficient material has been used. It is a less sensitive

method partly because five animals have been used per level instead of only two per level as for the mouse assay.

Two groups of women with amenorrhœa have been investigated separately (Table I). The first group consisted of six women whose excretion of gonadotrophins was in the normal range of menstruating women. Their urine was found to contain Gonadotrophin "A" only. The second group consisted of six women excreting gonadotrophins in high concentration. Five showed Gonadotrophin "A" only, and one Gonadotrophins "A" and "B".

Table I

MOUSE UTERUS ASSAY

Amenorrhœa

Gonadotrophins in range of normal cycle (<45 units/24 hr)

No of patients	*Gonadotrophins* A	B
6	+	—

Gonadotrophins in range of menopause (>45 units/24 hr)

No of patients	*Gonadotrophins* A	B
5	++	—
1	++	+

Five women who had passed the menopause have been examined (Table II). Their urine was found to contain a high concentration of Gonadotrophin "A". One preparation contained Gonadotrophin "B" and two others probably contained Gonadotrophin "B". In the latter only half the animals showed positive responses and there was not sufficient material for further assay

These results contrast with the urine in pregnancy. Eleven patients have been examined and it was found that Gonadotrophin "B" was generally present in high concentration (Table II) Gonadotrophin "A" was also present in urine

Table II

MOUSE UTERUS ASSAY

Menopause

No of patients	*Gonadotrophins A*	*B*
2	+++	−
2	+++	?+
1	+++	+

Pregnancy

No of patients	*Gonadotrophins A*	*B*
6	+	+++
4	−	+++
1	+	−*

*Showed a classical A-Z ovarian response

up to the third month of pregnancy, but not in International Standard Chorionic Gonadotrophin. In one case no Gonadotrophin "B" was detected by the uterus technique, which is relatively less sensitive to luteinizing substance, but the mice showed the classical Aschheim-Zondek ovarian response.

On three occasions the separate fractions from pregnancy urine were assayed by the prostate method. Some activity was observed in Gonadotrophin "A", but more than four times the amount in Gonadotrophin "B" on two occasions. It is possible that these columns were overloaded, since little is known yet of their capacity. The possibility of conjugated androgens being present must also be considered.

Discussion

These observations represent some of the results of a more comprehensive study of menstrual disorders of women. They are still incomplete and nothing more is claimed at present than that two gonadotrophic substances with different chromatographic properties can be recognized. Gonadotrophins "A" and "B" have not been demonstrated as different

hormones. They may be the same hormone attached to different proteins or, as Swingle and Tiselius have suggested in some of their work on other proteins, the second band may be material left as a tail behind the first band and subsequently picked up by more concentrated phosphate. It is remarkable, however, that the relative concentration of the two substances varies strikingly between the urine of pregnancy and the menopause; an observation that suggests that we are dealing with different hormones. If this is the case, we think we shall be justified in using an unspecific assay on the two fractions.

REFERENCES

DEKANSKI, J. (1949). *Brit J exp Path.*, 30, 272

LEVIN, L., and TYNDALE, H. H. (1936). *Proc. Soc. exp Biol., N.Y.*, 34, 516

LORAINE, J A. (1950) *J Endocrinol.*, 6, 319.

SWINGLE, S. M., and TISELIUS, A (1951). *Biochem. J.*, 48, 171.

DISCUSSION

LI: Have you mixed the menopausal and pregnancy urines together and put both on the column and still obtained these different bands?

BUTT· No, we have not mixed them.

LI You get your gonadotrophin B from pregnancy urine. If you put your B back on the same column, does it still come off as B? You don't get any more A?

BUTT. No, it all came out in B

LI. This would seem to indicate that A may be the follicle-stimulating factor.

LI It is generally known that menopausal urine is mainly follicle-stimulating. If the B corresponds to the luteinizing factor, then your menopausal urine has some luteinizing factor.

CROOKE· Yes, sometimes.

LI. And mainly luteinizing factor in pregnancy urine?

BUTT: Yes

ROBSON. Have you made any observations on the ovaries of these animals and tried to correlate your findings with the presence of these two fractions?

BUTT No, we've only worked with the uterus, except in one case I

mentioned where we had an AZ reaction in the ovary and no uterine response.

ROBSON: You should be able to correlate the exceptions in your A and B with exceptional changes in the ovary, if you really have two functionally different fractions.

ASTWOOD: Wouldn't you expect that if you did have pure luteinizing hormone of hypophyseal origin that you would get no response in the mouse uterine weight assay?

BUTT: All I can say is that we find that the mouse uterus is not very sensitive to chorionic gonadotrophin. We need a dose of at least 10 I.U. to be sure of a positive uterine response.

ASTWOOD: But I would think that in the case of pituitary luteinizing hormone there would be no response whatever. Is that not right, Dr. Li?

LI. No, I don't think so. If you inject either ICSH (as we call it) or pregnancy urine gonadotrophin, in normal mice you should get the œstrous uterus, but you do not get it in these hypophysectomized rats unless you inject immediately after operation. That is the differentiation which Prof Moricard emphasized When you inject gonadotrophin into an animal after hypophysectomy the picture is entirely different from that obtained by injection two weeks after the operation That is due

in the blood stream has disappeared. Then you have only the effect from the pregnancy urine gonadotrophin; that is to say, only *interstitial* cell hypertrophy, but no follicle formation.

R MORICARD: I was very surprised that you use the uterus for titrating the luteinizing factor. My assistant, Mlle Gothie, who is a biochemist, studied some years ago the effects of ultracentrifugation on chorionic gonadotrophin, and we obtained an extract that produced corpora lutea formation in the ovary of the immature mouse without

clinical conditions.

into saying

re most

gonado-

trophin?

BUTT: Yes, in pregnancy. But we have also recognized gonadotrophin B in the luteal phase of normal women.

LI: I would like to mention some recent experiments which Dr. Moon and I have done, using the highly purified follicle-stimulating hormone

in male mice. If we inject up to 12 5 mg. of our very pure follicle-stimulating hormone into normal mice, we get no microscopic change in the testes. In female mice the uterus showed greater sensitivity to hormone injections than did the ovaries.

SEGALOFF: Is it then follicle stimulating, Dr. Li?

LI. Yes, it is follicle-stimulating hormone. Probably our results with mice can be explained by a quantitative difference in response between mouse and rat ovary. Or it might need the presence of other hormones to form the follicles, perhaps thyroxine.

R. MORICARD: What is the response in the rabbit ovary when you inject the hypophyseal follicle stimulating factor?

LI: We have never injected rabbits with our purified fraction In the rat we know that it is purely follicle stimulating, having no effect on the uterus except in very high dosage.

ROBSON: Have you ever tried so-called pure luteinizing fraction in the hypophysectomized rabbit, and tried to maintain the corpus luteum? There you have an ideal test object for testing your so-called pure luteinizing hormone

LI. So far, there is no evidence that luteinizing hormone (interstitial cell stimulating hormone) *maintains* the corpora lutea

ROBSON: You suggest this is entirely different?

LI Yes I think Dr Astwood probably has some ideas on the maintenance of corpora lutea

ASTWOOD: If I speak to that question I'll use up my own paper for this afternoon.

PROBLEMS IN THE CLINICAL USE OF GONADOTROPHINS*

F. MORICARD

THE clinical use of gonadotrophins sets problems of dosage which are difficult to resolve. Gonadotrophins can induce follicle development in subcutaneous human ovarian grafts. A therapeutic result can be presumed from statistical study of pregnancies which have appeared in sterile subjects.

We have investigated especially the therapeutic action of equine gonadotrophin in human sterility.

History and Problem

In 1926, B. Zondek showed that hypophyseal implantations induce follicular development and œstrus in the immature mouse; in 1927, Aschheim produced proof of the elimination of gonadotrophins in the urine of pregnant women; and in 1930, Cole and Hart established the presence of a gonadotrophic hormone in the serum of pregnant mares.

In the therapeutic use of these gonadotrophins, the essential problem has been to determine what dose can induce follicle development in the human ovary. In 1935, R. Moricard produced an increase in the volume of ovarian grafts in the vulvæ in women, after an injection of chorionic gonadotrophin. In 1935, B. Zondek observed hæmorrhagic follicles in the ovary of a woman aged 30 who had received 26,500 rat units of chorionic gonadotrophin. In 1948, L. Claesson, B. Hogberg, Th. Rosenberg and A. Westman observed human follicles which developed after the injection of 36,000 international units of chorionic gonadotrophin. In the observations of B. Zondek and of L. Claesson a previous examination of the ovary could not be made.

*Université de Paris. Work of the Centre de Stérilité de la Clinique Gynécologique, Hôpital Broca, III rue L. M. Nordmann.

Our work was carried out on: (1) endocrine activity and periodicity of follicular development in human ovarian grafts (Douay's technique); (2) the posology of follicular development, induced especially by equine gonadotrophin, in sub-pubic ovarian grafts (study of nine cases); (3) the results of discontinuous and intermenstrual treatment of sterile women with equine gonadotrophin, injected in doses of about 2,400 international units.

Study of the Ovarian Cycle and Grafts in the Vulva

From 1929 to 1932, Douay, at the Gynæcological Clinic in Paris, recommended the systematic grafting of two pieces of ovary under the skin in the pubic region after hysterectomy, in order to avoid the troubles of castration In 1932 he was able to assemble 23 observations. He showed that if the woman was less than 35, a few months after the graft the neuro-vegetative disturbances of castration were disappearing, and the ovarian grafts enlarging periodically.

We have recorded that with functional ovarian grafts atrophy of the vulva following castration does not occur. Proof of follicular development in grafts can be demonstrated by puncture which withdraws some 3 ml. of follicular fluid. This fluid is at first yellow, but can become hæmorrhagic. In cases where grafts have been used in a woman with a uterus *in situ*, modifications of the mucosa of the body of the uterus occurred. In particular, a differentiation of secretory activity of the mucosa takes place during the luteal phase. This is proof of the luteal function of ovarian grafts. Follicular development in grafts takes place for only a few years, then ceases.

From the technical point of view, palpation allows the size of the ovary to be recognized, and puncture demonstrates the presence of the follicular growth.

Injections of Gonadotrophins and Stimulation of Follicular Development in Sub-pubic Ovarian Grafts

We have made an approximation between our rat units

and the international standard units, and can thus express approximately our results in international units.

In three patients in whom grafts were inserted in 1931 and 1932, after a period of spontaneous function, there was no development of follicles for three to six months. In these, development of follicles in the grafts could be stimulated by gonadotrophin treatment. This has been demonstrated by the presence of follicular fluid, withdrawn by puncture.

In one case, the doses used to stimulate follicular development were increased progressively. In 1934, it was possible to obtain follicular development with a dose of the order of 300 i.u. of chorionic gonadotrophin. In 1935, a dose of about 850 i.u. of chorionic gonadotrophin was necessary to obtain the same results. In 1936, with a dose of about 4,000 i.u. of equine gonadotrophin, two successive follicular developments were produced by one single hormonal treatment. In 1937, a dose of about 20,800 i.u. of equine gonadotrophin did not stimulate follicular development.

In six patients with grafts which had not yet been the site of periodic follicular development, a single gonadotrophin treatment incited successive follicular developments, which took place for 24 months, 20 months, 10 months, 6 months (two cases) and 5 months, respectively. The dose used varied between 300 i.u. chorionic gonadotrophin and about 12,800 i.u. equine gonadotrophin. The dose necessary to stimulate follicular development depended on the age of the graft.

These results indicate that a single treatment of 2,000–3,000 i.u. equine gonadotrophin is able to stimulate recurring follicular development for several months. The establishment of this fact has been the basis of a discontinuous hormone treatment with equine gonadotrophin in certain cases of sterility.

Discontinuous Intermenstrual Hormone Treatment of Certain Functional Cases of Sterility with Equine Gonadotrophin

The indication for hormonal treatment in unintentional

sterility is difficult to establish. It is necessary first to rule out a marital cause (absence of spermatozoa in the vagina, the cervix or the uterus), an anatomical cause (tubal patency negative), an infectious cause (exo- or endo-cervicitis), or an endocrine cause, as shown by repeated biopsy and menstrual examinations.

When it seems from the means at our disposal to be a matter of functional sterility, probably of ovular origin, it is impossible to demonstrate clearly whether there is: (*a*) absence of production of ovulæ, (*b*) absence of fertilization of the oocyte of the second order; (*c*) early degeneration of the fertilized egg; or (*d*) failure of implantation of the egg.

Out of 283 couples, sterile for more than two years, we selected 41 patients as being susceptible to gonadotrophic treatment. They were selected on the basis of sperm examination, tubal patency and luteal function (cytology of uterine mucosa) In 15 of these 41 women biopsy of the endometrium showed a slight anomaly of luteal function, indicated by the fact that the pre-menstrual endometrial biopsy after alcoholic fixation showed basal glycogen instead of the lucid zone (which could be interpreted as delay in the formation of the corpus luteum) or there existed a partial differentiation of connective spines (these are covered by epithelial cells and protrude into the lumen of the glands), or spiral arterioles were not present (which can be interpreted as indicating an insufficiency of progesterone secretion).

On the basis of temperature records and the appearance of the cervical secretion, injections are made between the 5th and 12th day of the menstrual cycle; 400 i.u. equine gonadotrophin* are injected intramuscularly each day for six days. The total dose injected is usually 2,400 i.u.; the minimum for this series of patients was 1,200 i.u. and the maximum 3,200 i.u. At doses of 1,000 to 5,000 i.u. equine gonadotrophin the treatment is well tolerated, but with higher doses we

*[illegible]

have observed some side effects (asthenia, hyperthermia). An interval of two to three months is left between each treatment.

From 41 cases of sterility treated in this way, we have obtained 22 pregnancies and 19 failures. Of the 15 women with abnormal luteal function included in this group, there were 9 pregnancies, and 6 failures. Of the pregnancies· 10 women became pregnant in the first month of treatment; 6 women menstruated again after the treatment before becoming pregnant; 4 women menstruated twice after the treatment before becoming pregnant; 1 woman had 4 menstrual periods, and another 5 before becoming pregnant. Of particular interest were the 12 women who remained sterile during and immediately after the gonadotrophin treatment and had one or more menstruations before pregnancy occurred.

The results obtained indicate that discontinuous and intermenstrual gonadotrophin treatment stimulates: first, follicular development (more or less abnormal); then, in the following cycles, the production of an oocyte of the second order, giving an egg capable of segmentation and of normal implantation, provided there is good luteal function. This can explain the time which separates hormonal treatment and the appearance of pregnancy.

Conclusions

We have established that one single hormonal treatment, injection of equine gonadotrophin, can stimulate (at least in ovarian grafts in the vulva) periodic development of follicles, continuing for several months. Out of 283 cases of sterility, after investigating spermatic, tubular and luteal functions, we have treated 41 patients with a discontinuous and intermenstrual treatment of 1,200–3,200 i.u. equine gonadotrophin. In these 41 patients we have obtained 22 pregnancies. These results can be interpreted as showing that injected equine gonadotrophin produces several successive developments of follicles, one of which is accompanied by the production of an oöcyte of the second order, with the

formation of an egg whose fertilization is followed by a normal pregnancy.

REFERENCES

CLAESSON, L., HOGBERG, B., ROSENBERG, TH., and WESTMAN, A. (1948). *Acta endocrinol.*, **1**, 1.

DOUAY, E. (1932). *Auto greffes ovariennes.*, Bizou, A. Arnette, Paris.

MORICARD, F. (1943) *Hormonologie sexuelle humaine.* Paris· Masson.

ZONDEK, B. (1935) *Hormone des Ovariums und des Hypophysenvorderlappens*, p 522.

DISCUSSION

the

ch

ROBSON: That's even more important, isn't it, than the histological changes?

F. MORICARD: No, by studies of the uterine mucosa I wanted to prove that we have secretion of œstrogen from the follicles and then progesterone. You can have menstruation without the formation of corpora lutea.

ASTWOOD Might I ask whether the presence of the uterus modified the graft in any way?

F. MORICARD: Not immediately

R MORICARD: In a case in which there was bilateral ovariectomy and

In the grafted ovaries we have generally only a growth of the follicles, and it seems that the formation of corpus luteum may occur, but not generally.

GADDUM What was the longest time these grafts went on working?

F. MORICARD About three years. It depends on the age of the patient. When the patient is very young the grafts function much longer

CLINICAL APPLICATIONS OF THE ASSAY OF PITUITARY AND PLACENTAL GONADOTROPHINS

J. A. LORAINE

THE gonadotrophic substances elaborated by the anterior pituitary are the Follicle-Stimulating Hormone (FSH), the Luteinizing Hormone (LH) and Luteotrophin (LTH). As with other anterior lobe hormones, modern advances in our knowledge of the actions of pituitary gonadotrophins have stemmed from two main events: firstly, from the development and perfection of techniques of hypophysectomy in experimental animals, demonstrating unequivocally the intimate relationship of the pituitary to gonadal function; and secondly, from the chemical fractionation, purification and characterization of the individual gonadotrophic substances. In the elucidation of the chemical nature of these hormones, mention should be made of the work of Fevold (1939), of Li, Simpson and Evans (1940) and of Greep, Van Dyke and Chow (1942). These substances are now known to be proteins of high molecular weight and to contain sugar in their molecules, for which reason they are commonly referred to as glycoproteins. Their actions have been studied extensively in normal and hypophysectomized animals.

Assay of Pituitary Gonadotrophins in Clinical Practice

FSH and LH are known to occur naturally in humans and are the gonadotrophic substances estimated in men, non-pregnant women and children. At present no available assay method is sufficiently sensitive to detect these hormones in blood and so far all studies have been conducted on urine, extracted and concentrated by a variety of procedures.

Most investigators have used either rats or mice as test animals. Ideally assays should be performed on hypophysectomized animals, so that the endogenous production of gonadotrophins from the animal's own pituitary does not interfere with the results.

Present methods of assay of FSH and LH are rather unsatisfactory for a number of reasons. In the first place, no international standard is available for comparison with unknown preparations, and this necessitates expression of results in arbitrary "rat" and "mouse" units. These animal units are notoriously variable from laboratory to laboratory, and are also very unsatisfactory from the statistical point of view. Secondly, estimations are made, not of a single substance, but of at least two different substances, present in varying concentrations at different times. Finally, the quantities present in urine are usually very small, and elaborate and tedious methods of preparation and concentration are usually required. Such procedures may lead to considerable loss of gonadotrophic activity and may render the final extracts toxic to the experimental animals.

The extraction methods used have included alcohol precipitation (Heller and Heller, 1939; Smith, Albright and Dodge, 1943), acetone precipitation (Frank and Salmon, 1935), ultra filtration (Gorbman, 1945; Jungck, Maddock and Heller, 1947), tannic acid precipitation (Levin and Tyndale, 1937, Levin, 1941), and kaolin adsorption (Loraine, 1950*a*; Dekanski, 1949, McArthur, 1952). In this laboratory the method involving adsorption on kaolin, elution with N/10-NaOH and subsequent precipitation by acetone has proved convenient and satisfactory for the preparation of FSH and LH from urine.

Of the very numerous assay methods employed for FSH and LH estimations in clinical practice, it is proposed to mention only two. These are the mouse uterus test and the test involving enlargement of the ventral prostate in hypophysectomized rats.

An assay method which has gained wide popularity in

America for the assay of FSH depends on the *enlargement of the uterus in intact immature mice.* Most centres use the technique described by Klinefelter, Albright and Griswold (1943) or some modification thereof; results are expressed in "mouse uterine units" of FSH per 24 hours, a unit being defined as that quantity necessary to produce a given effect. *This method is a very sensitive one, and gives positive results* with most urines It has been of undoubted value to the clinician in differentiating primary gonadal failure from gonadal failure secondary to lesions of the pituitary. In primary gonadal failure, e.g., ovarian agenesis (Turner's syndrome) in females or sclerosing tubular degeneration (Klinefelter's syndrome) in males, large amounts of gonadotrophin appear in the urine. The pituitary is apparently *striving to stimulate unresponsive gonads.* In pituitary failure, e.g., Simmonds' disease, on the other hand, quantities excreted are very small indeed and gonadotrophic activity may not be detectable even by this sensitive method. Howard, Sniffen, Simmons and Albright (1950) on the basis *of this test have classified cases of eunuchoidism in man into* two groups those with a high urinary excretion of FSH and those with a low or normal excretion of FSH. Most cases fall into the second category

It should be emphasized, however, that the mouse uterus *test is at best a qualitative rather than a quantitative assay* procedure, and little or no reliance can be placed on minor fluctuations in gonadotrophin excretion in individual patients. Moreover, it scarcely seems justified to equate the response of the mouse uterus in FSH units. Perhaps the term "total *gonadotrophins" would be more appropriate.*

According to Greep, Van Dyke and Chow (1942), the *enlargement of the ventral lobe of the prostate in hypophysectomized rats* is a unique test, in that it is specific for LH and is not affected by FSH. Recently this technique has been applied by McArthur (1952) as a method of assay of LH suitable for clinical use. This method has a degree of sen-

test. Extracts prepared by the kaolin method are tested for their FSH activity by the increase in testicular weight in hypophysectomized rats and also by histological changes in the testes. Preliminary studies using this method suggest that menopausal women excrete both FSH and LH. In normally menstruating women there are two peaks of LH excretion, one occurring at approximately mid-cycle and the other in the late luteal phase just prior to the next menstrual period.

Other methods of assay of LH attempted recently include: radioactive phosphorus uptake of the ventral prostate (Taymor and McArthur, 1952) and acid phosphatase concentration of the prostate in hypophysectomized rats (Schaffenburg and McCullagh, 1951)

Bioassay of Chorionic Gonadotrophin

This hormone is elaborated by the chorionic villi of the human placenta during pregnancy and is found in large quantities in the blood and urine of pregnant women, and also in such conditions as hydatidiform mole and chorion-epithelioma, where actively proliferating chorionic tissue is present. CG, in common with other gonadotrophins, is of protein nature and belongs to the group of glycoproteins. The hormone has recently been isolated in a purified state (Katzman, Godfrid, Cain and Doisy, 1943, Claesson, Hogberg, Rosenberg and Westman, 1948) CG has a predominantly luteinizing action, but it should be emphasized that it is a different chemical substance from pituitary LH and should not be confused with it.

An international standard is available for comparative assay of CG, and all results should now be expressed in international units By definition, 1 i.u. is the activity contained in 0·1 mg. of the standard preparation. The main source of uncertainty in assays of CG has always been the fact that each colony of animals is liable to differ in sensitivity from other colonies. This error is largely eliminated by the use of a standard preparation. There is no longer any excuse

for estimating CG in terms of arbitrary "rat" and "mouse" units. Some of the methods used for the bioassay of CG are shown below.

METHODS OF ASSAY OF CHORIONIC GONADOTROPHIN

Primary effects	*Secondary effects*
Corpora lutea (rats, mice) Ovarian weight (rats, mice) Ovarian *hyperæmia (rats)* Ovulation in rabbits Ovulation in female toads (*Xenopus lævis*) Spermatogenesis in male toads	Uterine weight (rats) Vaginal smears in intact or *vitamin B deficient rats* Seminal vesicles (rats) Prostatic weight (rats)

Tests can be divided according to whether the effect is primary or secondary In the primary group the organs inspected are the gonads themselves, while in the secondary group the effects result from liberation of œstrogens or androgens from the gonads. Many of these methods have considerable disadvantages when applied to the quantitative assay of CG in the blood and urine of patients, and it is unfortunate that many early investigators in this field estimated CG by inaccurate and unreliable methods of assay. This subject has recently been reviewed (Loraine, 1952). In the last few years, the two methods which have been applied most extensively in the clinical field are those depending on ovarian hyperæmia and prostatic weight in rats.

The *ovarian hyperæmia* test introduced by Zondek and Sulman (1945) for the diagnosis of pregnancy has been made the basis of an elegant clinical bioassay of CG by Albert and co-workers at the Mayo Clinic (Albert, 1948; Albert and Berkson, 1951). This method is sensitive and is reasonably accurate from the statistical point of view. In addition, the total time required for the test is only 4 hours, making it a bioassay unique in its rapidity. This is in marked contrast to most other procedures involving multiple injections into rats and necessitating 72- and 96-hour injection periods. Unfortunately, however, the success of the ovarian hyperæmia

method appears to be conditioned to some extent by the strain of animal employed. In other laboratories the technique could not be made the basis of a satisfactory quantitative assay of CG.

The method depending on the *enlargement of the prostate* in immature rats (Loraine, 1950*b*) has been shown to be an accurate and sensitive method for the quantitative assay of CG in the blood, urine and placentæ of patients. In addition, the œstrogens in pregnancy urine do not interfere with the test, and accordingly untreated urine may be employed for the assays. The clinical studies subsequently discussed have been made using the prostatic weight method.

Clinical Applications of CG Assay

The value of CG estimations in the diagnosis of pregnancy and in cases of hydatidiform mole and chorion-epithelioma is well known. No further reference will be made to these conditions.

Normal Pregnancy

CG curves in international units are now available for both the urinary excretion (Loraine, 1950*b*; Albert and Berkson, 1951) and for the serum concentration (Jones, Delfs and Stran, 1944; Wilson, Albert and Randall, 1949; Loraine and Matthew, 1952). Using the prostatic weight method, it has been shown that in the first trimester of normal pregnancy, very high readings, e g. 20,000–50,000 I.U., are obtained for the urinary excretion (I U per 24 hours), and the serum concentration (I.U. per litre). In the second and third trimesters, however, the urinary excretion and serum concentrations are in the range 4,000–11,000 I.U. ($P = 0 \cdot 99$). Estimations of CG consistently outside this range should be regarded as pathological.

Pre-Eclamptic Toxæmia

Interest has chiefly been centred on the possible value of urinary and serum CG estimations as a prognostic index in

such cases (Smith and Smith, 1934, 1939, 1941, 1948; Taylor and Scadron, 1939; Watts and Adair, 1943; Loraine and Matthew, 1950). The Smiths, using corpora lutea in the ovary in rats to assay CG, were the first to demonstrate that a proportion of pre-eclamptic patients (over 70 per cent of their series) showed an abnormally high serum concentration of CG when compared with normal pregnant women They further suggested that a rise in the serum CG might be regarded as a warning of impending toxæmia. This latter finding was not confirmed by later investigators.

Using the prostatic weight method of assay, Loraine and Matthew (1950) studied the serum concentration and urinary excretion of CG in 29 cases of pre-eclamptic toxæmia, nine cases of essential hypertension in pregnancy, and five cases of essential hypertension with superimposed toxæmia. The pre-eclamptic patients were further subdivided into three groups—severe, moderate and mild. The assessment of the severity of the toxæmia was based mainly on the rapidity of evolution of the disease and on its response to therapeutic measures. In severe pre-eclamptic toxæmia the mean urinary excretion and serum concentration of CG were significantly higher than in normal pregnancy, but in none of the other groups of patients was this observed. No correlation could be found between the high CG readings and any particular clinical feature such as blood pressure, albuminuria, or œdema It was therefore concluded that at the present time routine assay of the urinary and serum CG would not be of any great assistance to the clinician in the management of these cases.

Diabetic Pregnancy

Time does not permit of a detailed consideration of the prognostic value of hormone estimations in diabetic pregnancy. It will be remembered that Priscilla White and her co-workers (1946, 1949) used a falling pregnanediol excretion and a rising serum CG concentration as prognostic criteria in pregnant diabetics. In cases with normal findings, the fœtal survival

rate was 97 per cent, whereas with abnormal CG and pregnanediol readings the incidence of live births was only 52 per cent. The administration of stilbœstrol and progesterone in gradually increasing dosage throughout pregnancy corrected this so-called "hormonal imbalance" by producing consistent depression of CG titres. In the patients receiving such therapy the fœtal survival rate rose to 90 per cent and the incidence of pre-eclampsia was greatly reduced.

In this laboratory it has been demonstrated that a proportion of pregnant diabetic patients have abnormally high concentrations of CG in blood and urine, but it has not been possible to confirm any of White's other findings. No correlation could be obtained between the urinary excretion and serum concentration of CG on the one hand, and any medical or obstetrical finding on the other; no evidence could be obtained that elevation of the CG titre in blood and urine could be regarded as the herald of an impending obstetrical disaster. Indeed, in the 40 patients so far studied, fœtal death appeared just as likely to occur in patients with normal CG values as in those with abnormally high figures. In addition, stilbœstrol produced only an evanescent fall in the 24-hour excretion of CG in normal and diabetic women (Loraine, 1949). Consistent depression of the urinary CG titre by prolonged œstrogen therapy was not obtained even when large doses of stilbœstrol (100–120 mg./day) were employed The initial depression was soon followed by an "escape" phenomenon, the readings tending to climb back to their original level while the patient was still under therapy. This effect is shown in a normal patient in Fig. 1 and in two pregnant diabetics in Figs. 2 and 3.

Fig. 4 shows the urinary excretion of CG in 25 pregnant diabetics who did not receive hormonal therapy.

Renal Clearance of CG

As mentioned previously, the urinary excretion and serum concentration of CG fluctuate characteristically during normal pregnancy. This fluctuation might represent alterations in

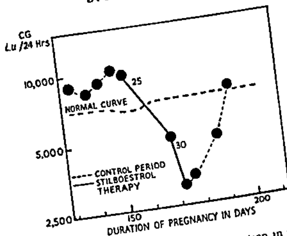


Fig. 1 Effect of stilbœstrol on the CG excretion in a normal pregnant woman The figures on the curves denote doses (mg. per day) and are placed at the times these were started.

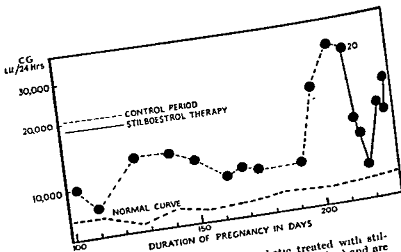


Fig 2. CG excretion in pregnant diabetic treated with stilbœstrol The small figures denote doses (mg. per day) and are placed at the times these were started.

the rate of formation, in the rate of destruction or in the rate of excretion of the hormone. In an attempt to elucidate this problem, the renal clearance of CG under normal and pathological conditions has been studied by Gastineau, Albert and Randall (1949), using the ovarian hyperæmia method of assay, and by Loraine (1950c), employing the prostatic weight test. Clearance was estimated by the formula UV/B and was expressed in ml./minute.

Estimations of CG clearance were made in 12 normal

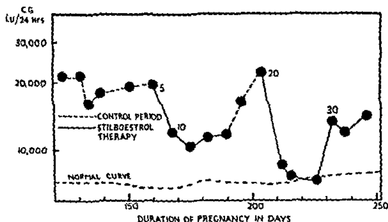


FIG. 3 CG excretion in pregnant diabetic treated with stilbœstrol The small figures denote doses (mg. per day) and are placed at the times these were started.

pregnant women, 29 cases of pre-eclamptic toxæmia, seven pregnant diabetics, nine cases of essential hypertension in pregnancy, and five cases of essential hypertension with superimposed toxæmia (Loraine, 1950c). In all types of case the mean clearance of CG was found to be less than 1·00 ml. per minute. In normal pregnancy the mean figure was 0·95 ml. per minute. In normal and diabetic pregnancy constant figures for clearance were obtained in the three trimesters of pregnancy, and accordingly it was concluded that variations

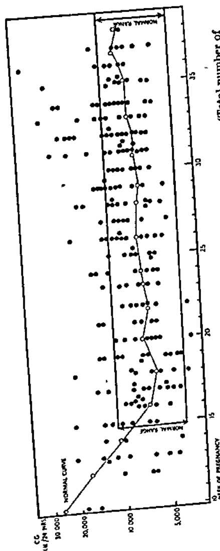


FIG. 4. CG excretion in 25 pregnant diabetics without hormonal therapy. (Total number of estimations, 230.)

the rate of production or destruction of the hormone and did not arise from differences in the renal excretion.

The pre-eclamptic patients were again classified into severe, moderate and mild groups (Loraine and Matthew, 1950). In the mild and moderate cases the mean clearance did not differ significantly from that in normal pregnancy ($P=0 \cdot 99$). Similar results were obtained in cases of essential hypertension in pregnancy and in essential hypertension with superimposed toxæmia. In the severe pre-eclamptic group and in the pregnant diabetics, however, the mean clearances (0·74 and 0·75 ml./min. respectively) were significantly lower than those in normal pregnancy.

REFERENCES

ALBERT, A (1948) *J. clin. Endocrinol.*, **8**, 619.

ALBERT, A., and BERKSON, J. (1951) *J. clin. Endocrinol.*, **11**, 805.

CLAESSON, L, HOGBERG, B, ROSENBERG, T., and WESTMAN, A. (1948). *Acta endocrinol*, **1**, 1.

DEKANSKI, J. (1949). *Brit. J. exp Path.*, **30**, 272.

FEVOLD, H. L. (1939). *J. biol. Chem.*, **128**, 83.

FRANK, R. J., and SALMON, U. J (1935). *Proc. Soc. exp. Biol. Med.*, **32**, 1237.

GASTINEAU, C. F., ALBERT, A., and RANDALL, L. M. (1949). *J. clin. Endocrinol*, **9**, 615.

GORBMAN, A. (1945). *Endocrinology*, **37**, 177

GREEP, R. O., VAN DYKE, H. B, and CHOW, B. F. (1942). *Endocrinology*, **30**, 635.

HELLER, C. G., and HELLER, E. J (1939). *Endocrinology*, **24**, 319.

HOWARD, R. P., SNIFFEN, R. C, SIMMONS, F. A, and ALBRIGHT, F. (1950) *J clin. Endocrinol.*, **10**, 121.

JONES, G E S., DELFS, E., and STRAN, H. M. (1944). *Johns Hopk. Hosp. Bull*, **75**, 359.

JUNGCK, E. C., MADDOCK, W. O., and HELLER, C. G. (1947). *J. clin. Endocrinol*, **7**, 1.

KATZMAN, P. A., GODFRID, M., CAIN, C. K., DOISY, E. A. (1943). *J. biol. Chem.*, **148**, 501.

KLINEFELTER, H. F., Jr., ALBRIGHT, F., and GRISWOLD, G. C. (1943). *J. clin. Endocrinol.*, **3**, 529

LEVIN, L. (1941) *Endocrinology*, **28**, 378.

LEVIN, L., and TYNDALE, H. H. (1937) *Endocrinology*, **21**, 619.

LI, C. H., SIMPSON, M E, and EVANS, H. M (1940). *Endocrinology*, **27**, 803

LORAINE, J. A. (1949). *Brit med. J*, ii, 1496.

LORAINE, J A. (1950a) *J. Endocrinol.*, **7**, ii

LORAINE, J. A. (1950*b*). *J. Endocrinol.*, **6**, 319.
LORAINE, J. A. (1950*c*) *Quart. J. exp Physiol.*, **36**, 11.
LORAINE, J. A. (1952). *J. Obstet. Gynæc., Brit. Emp.*, **59**, 536.
LORAINE, J. A., and MATTHEW, G. D. (1950) *J. Obstet Gynæc., Brit Emp.*, **57**, 542.
LORAINE, J. A., and MATTHEW, G. D. (1952) Unpublished observations
MCARTHUR, J W. (1952) *Endocrinology*, **50**, 304
SCHAFFENBURG, C A., and MCCULLAGH, E. P (1951). *J. clin. Endocrinol*, **11**, 765
SMITH, G. V., and SMITH, O. W (1934) *Amer. J. Physiol*, **107**, 128
SMITH, G. V., and SMITH, O. W. (1939). *Amer. J Obstet Gynec.*, **38**, 618
SMITH, G. V., and SMITH, O. W. (1941) *J. clin. Endocrinol.*, **1**, 470
SMITH, G. V., and SMITH, O. W (1948). *Physiol. Rev.*, **28**, 1.
SMITH, P. H., ALBRIGHT, F, and DODGE, E. (1943). *J. Lab. clin. Med*, **28**, 1761.
TAYLOR, H. C, and SCADRON, E. N. (1939) *Amer J Obstet. Gynec*, **37**, 963.
TAYMOR, M, and MCARTHUR, J W. (1952). Personal communication.
WATTS, R. M., and ADAIR, F. L (1943) *Amer. J Obstet Gynec.*, **46**, 183
WHITE, P (1946) In *Joslin's Treatment of Diabetes Mellitus*, 8th ed, p 769. London: Kimpton
WHITE, P. (1949). *Amer J Med.*, **7**, 609.
WILSON, R B., ALBERT, A, and RANDALL, L. M. (1949) *Amer. J Obstet. Gynec*, **58**, 960.
ZONDEK, B., and SULMAN, F (1945) *Vitam. & Horm.*, **3**, 297.

DISCUSSION

GRAY Miss Wood, working in my department at King's College Hospital, has carried out a series of urinary chorionic gonadotrophins

are the patients consistently high, or do they bounce in and out of the normal range, or what happens?

LORAINE Some patients start off normally and the excretion rises at

pregnancy.

GADDUM: A large-scale experiment has been started in this country—it's gone on for some time now—with statistical controls and placebos. Eventually this experiment should give definite information on that point.

One point in connection with Dr. Loraine's results that intrigued me was that the concentration of CG in the blood was always nearly equal to the concentration in the urine. In 7 groups of patients the average ratios of the two concentrations all lay between 0 86 and 1·26—the average of the averages was 1 03. It has always been a puzzle to me how the kidney manages to do it There are three obvious possibilities: (1) There is no CG in the glomerular filtrate and the tubules excrete CG till the concentration in the urine is equal to that in the blood (2) Small amounts of CG in the filtrate are concentrated to just the right amount by the absorption of water. (3) The concentrations of CG in the filtrate and in the reabsorbed fluid are equal to that in the blood. This last possibility is the easiest to understand but perhaps not the easiest to believe

SEGALOFF: May I ask another question, relative to the assay of pituitary gonadotrophins The longer I have worked in this field the more I am confused, and today even more than before What is FSH? Is there such a thing? And do we have a straightforward assay for it? Dr Li has told us that he has injected *pure* FSH, which I presume then must be FSH, and in the mouse it is inactive. On the other hand, Dr Loraine has mentioned evaluating the content of FSH by the microscopic picture of the rat testis And yet, reports in the literature from Dr. Li and his co-workers tell us that LH produces complete develop-

LORAINE Dr McArthur injects the animals 4 days after hypophysectomy

LI Then there is still some FSH present—it's there for some time

With regard to the data I mentioned about mice, the mouse is a very tough animal with which to get any notable increase in any activity

lating hormone. Why this is so, we don't know, but we want to find out. In the hypophysectomized animal, when you inject FSH all you

distinct reaction in hypophysectomized male and female rats.

LORAINE: Do you think, Dr. Li, that this method of LH assay pro-

hypophysectomized animal, or the ovary. Of course the sensitivity there might be very low, because you cannot titrate. All you can do is observe the lowest effective dose which will produce repair of the tissue of the interstitial cells in both testes and ovary. In such cases there is no synergism between FSH and ICSH—only the ICSH would be involved in repair.

ROBSON: There's one point I'd like to put here very tentatively, that it is becoming quite clear that the reactivity of an organ like the ovary or the testis changes after hypophysectomy. For example, if you hypophysectomize the rabbit and you inject any kind of gonadotrophic hormone, within the next few days you can get follicular growth, ovulation and luteinization. But if you wait a longer time then the reactivity

the reactivity of different components of a gonad may change at different speeds after hypophysectomy, and it is possible that the various reactions which are observed and which are taken as specific reactions of different gonadotrophins might be explained as reactions of tissues whose reactivity has changed differentially after hypophysectomy.

LI: Yes, we have recently been doing some work on the thesis that the main component responsible for this sensitivity or responsiveness

hypophysectomized rabbits, and then the reactivity of the ovaries was

maintained and the original amount of gonadotrophin injected intravenously would produce ovulation.

R. Moricard: In connection with Dr Li's remark about thyroxine, I [illegible]

plays a part.

TESTS FOR LUTEOTROPHIN

E B. ASTWOOD

THE term luteotrophin has been used to designate th hypophyseal factor essential for the maintenance of functio of the corpus luteum in the non-pregnant animal. In mos forms hypophysectomy is followed by a prompt regressio of the corpus luteum while in some species, including the ra (Smith, 1930), corpora lutea persist and, though *functionless* retain a fairly normal cellular structure. Under these cir cumstances the administration of concentrates of follicle stimulating hormone or of luteinizing hormone fails to evok the secretion of corpus luteum hormone (Greep, 1938) Indeed certain luteinizing hormone preparations (Greep 1938), but not others (Greep, Van Dyke and Chow, 1942) destroy the persisting corpora lutea of hypophysectomize rats. *However, pituitary extracts free of follicle-stimulating* and luteinizing hormones can be prepared which will caus further luteal growth and sustain luteal function after hypophysectomy.

Hypophyseal luteotrophin is to be distinguished from the hormones of placental origin which extend the functional life of the corpus luteum in pregnancy. These "chorionic luteotrophins" are largely species specific; they differ physiologically and chemically from each other and from the pituitary hormone, and are outside the scope of this brief review.

Before considering the tests which *might* serve as a basis *for* the assay of luteotrophin, certain other factors which influence luteal function should be recalled. *The corpus* luteum of the rabbit appears to differ from *that of other* mammals which have been investigated in responding to the direct action of œstrogen. The initial observations of Robson

(1937) and of Westman and Jacobsohn (1937) that œstrogen would sustain luteal function in this species have been amply confirmed (Heckel and Allen, 1939; Greep, 1941). The effect is clearly not mediated by the hypophysis; indeed Hammond and Robson (1951) have recently shown a direct action of œstrogen implanted into the corpus luteum, in the form of a crystal or a pellet, upon contiguous luteal tissue. While œstrogen seems to promote luteal function by an effect upon the hypophysis in other species, a direct action upon the corpus luteum has been shown only in the rabbit. In some species removal of the uterus seems to induce a prolongation of luteal function. Since the original observation of Loeb (1923) in the guinea pig, a similar phenomenon has been encountered in the rabbit (Asdell and Hammond, 1933; Greep, 1941) and perhaps even in the rat (Bradbury *et al.*, 1950). The mechanism here is singularly obscure.

No entirely satisfactory method has been proposed for the assay of luteotrophin. The tests which have been used are time consuming and at best only semiquantitative, some lack specificity and are subject to interference by other pituitary hormones. An assay method for a hormone should be one which is easily and quickly carried out and is quantitatively accurate. The best methods are those which depend upon a prompt response to a single injection, such as the blood sugar response to insulin, the adrenal ascorbic acid depletion assay for corticotrophin, the antidiuretic test for the pressor principle, and the ovarian hyperæmia test for chorionic gonadotrophin. When repeated and prolonged administration is required, as in tests for growth hormone, progesterone, androgens, and follicle-stimulating hormone, the degree of response is strongly influenced by the steadiness of action of the test substances, and the result may be greatly modified by the frequency and route of administration, as well as by the presence of accessory substances which alter the rate of absorption Most tests for luteotrophin are complicated by these factors, and there is reason to believe that they are even more important in this instance than in others. The

corpus luteum exhibits the peculiar property of losing its responsiveness to luteotrophin if it is deprived of it for some hours. If too long an interval elapses after hypophysectomy before luteotrophin treatment is begun, or if a lapse in treatment occurs, the corpus luteum undergoes irreversible changes and becomes refractory to further stimulation. Furthermore, the corpus luteum appears to have a self-determined and limited life-span beyond which luteotrophin cannot prolong its function. For this reason, attempts to prolong the luteal phase in those species whose cycles normally are attended by a progestation phase, or attempts to prolong the pseudopregnancy phases of other animals, have failed. It becomes necessary, therefore, to resort to replacement therapy after hypophysectomy or to make use of the abortive luteal phases of animals such as the rat.

The Question of the Identity of Prolactin, Lactogenic Hormone, and Luteotrophin

Currently it is a widely held view that prolactin (the factor responsible for the crop-gland reaction in the pigeon), lactogenic hormone (the pituitary principle initiating milk secretion in mammals), and luteotrophin are one and the same thing. Though this may very well prove to be the case, the evidence cannot at present be regarded as conclusive.

The strongest support for the view that a single substance mediates these three widely different effects derives from the fact that prolactin preparations (Lyons, 1937) that are purified to the extent of behaving as homogeneous proteins in solubility studies and electrophoresis, are highly active by all three tests. Recent experience with so-called pure corticotrophin and crystalline, electrophoretically homogeneous growth hormone suggests caution in accepting this type of evidence as final. The "pure" corticotrophin protein can readily be fractionated by adsorption on cellulose or oxycellulose to yield a highly active fraction and an inert protein which still meets the same criteria of purity as the original protein (Sayers and Astwood, 1951) and preparations of

corticotrophin 100 times as active as the "pure protein" can readily be prepared. Even at this stage of purification certain corticotrophin concentrates are heavily contaminated with intermedin, adipokinin, and perhaps other factors (Raben, Rosenberg and Astwood, 1952). Similarly, "pure" growth hormone is apparently contaminated with one or more factors which account for the diabetogenic and adipokinetic effects which are generally regarded as properties of the growth-promoting factor itself (Raben, Westermeyer and Leaf, 1952; Rosenberg, 1952).

Physiological considerations and a limited amount of experimental evidence suggest that crop stimulation, lactogenesis, and corpus luteum maintenance may not all be attributable to a single substance. Lactation and a functioning corpus luteum may sometimes coexist, as in the normal rat, but usually periods of luteal function, such as the luteal phase of the sex cycle, pseudopregnancy, and pregnancy, are not associated with mammary secretion. Rather, lactation seems in some way to be initiated by a cessation of luteal function.

The early experiments of Nathanson and Fevold (1938) indicated that not only prolactin preparations, but concentrates of luteinizing hormone free of prolactin, as well as extracts of postpartum urine containing little of either, could prolong luteal function in the normal mouse. Initially Evans, Simpson, and Turpeinen (1938) found that luteal function could be maintained by extracts rich in either corticotrophin or prolactin, and only later (Evans, Simpson, Lyons, and Turpeinen, 1941) identified the active principle with prolactin. While crude pituitary extracts are known to initiate milk secretion in normal animals, it has not yet been clearly shown that the crop gland stimulating hormone is the factor involved.

As long as there is any question as to the identity of luteotrophin with prolactin or the lactogenic factor, it would be desirable to have a simple and accurate assay related to its physiological action on the mammalian corpus luteum.

Prolongation of the Œstrous Cycle in Normal Animals

It has been known since the original observations of Long and Evans in 1922 that crude pituitary extracts interrupt the œstrous cycle of rats and lead to the formation of many large corpora lutea which can be shown to be functioning (Teel, 1926). The observation of Dresel (1935) that preparations of prolactin temporarily interrupt the œstrous cycle of the mouse has been confirmed (Nathanson and Fevold, 1938) and extended to the rat (Lahr and Riddle, 1936; Tobin, 1942). Tests based upon this phenomenon cannot be regarded as entirely specific, as other hormones, many toxic agents, and various stressful situations are known to disturb the œstrous cycle and lead to anœstrus or occasionally to a state of pseudopregnancy

With proper precautions, however, a reliable test can be performed in normal cyclic rats By selecting animals with regular four- or five-day cycles, as judged by observation for a two- to three-week interval, one can predict with a probability of better than 95 per cent that the cycle under observation will be of the customary length if no treatment is given A potent preparation administered during the day after the first œstrous smear will prolong a four-day cycle by one day. If the treatment is continued for two days, the cycle will be extended to a six-day cycle; three days, a seven-day cycle; and so on. Injections during the first day of the œstrous smear do not alter the cycle, presumably because the corpus luteum is already functioning during that short interval. If treatment is delayed until two days after the first day of the œstrous smear, the cycle will not be delayed; in this case, it is presumed that the corpus luteum has already become non-functional and its activity cannot be restored (Fig 1).

As a practical test one can administer the luteotrophic extract for one to three days, beginning 24 hours after the first appearance of the cornified smear, and expect as a positive result a corresponding delay of one to three days in the appearance of the next œstrous. Should the next cycle

not appear on the predicted day, but be further postponed, the test becomes invalid; the animal may have become pseudopregnant or some non-specific, painful or toxic agent may have induced anœstrus. If the dose of the luteotrophic extract is too small, or the injections spaced at too long

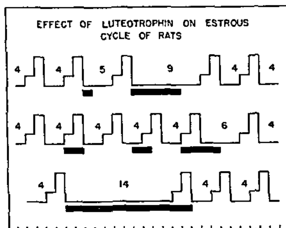


FIG 1 Effects of twice-daily injections of a luteotrophic preparation for from one to fourteen days upon the four-day œstrous cycle of the rat It may be noted that when injections were started on the day after vaginal cornification, treatment for one and for five days extended the cycles to five and to nine [illegible]

intervals, the expected delay of the next cycle will not be realized. Such a test would seek to define the minimal effective dose, but the result would be modified by the frequency of injection, the presence or absence of substances which alter the rate of absorption, and by the presence of follicle-stimulating, luteinizing, and perhaps other hormones in the extracts being tested.

Inhibition of the Œstrous Vaginal Smear in Œstrogen-treated Hypophysectomized Rats

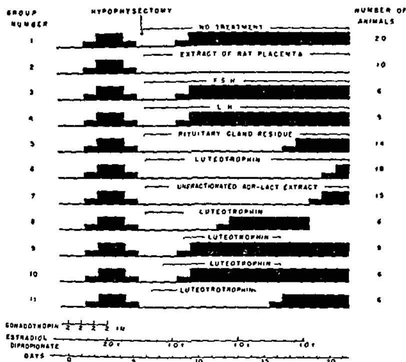
When normal cyclic female rats are treated with œstrogen, the immediate vaginal œstrous response gives place to a "diœstrous" smear; corpora lutea persist and enlarge and their secretion converts the cornified vaginal mucosa into the "mucified" condition typical of pregnancy, and the vaginal smear resembles that of diœstrus. If the ovaries are removed or if corpus luteum function is interrupted by hypophysectomy while œstrogen is continued, vaginal œstrus ensues in two to three days. Luteotrophic preparations may thus be tested by determining the dose required to prevent a return of vaginal cornification following hypophysectomy (Nelson, 1946) Immature rats can similarly be employed by first inducing the formation of corpora lutea by treatment with chorionic gonadotrophin. Concurrent and continued treatment with œstrogen permits the inhibitory action of corpus luteum hormone to be detected by daily vaginal smears. As *in the adult animal, hypophysectomy is followed by a return* of vaginal cornification, while the daily or twice-daily injection of luteotrophin will sustain luteal function as shown by the "diœstrous" smear (Fig 2) (Astwood, 1941) This test, while more specific, suffers from the same quantitative limitations as the tests based upon prolongation of the œstrous cycle.

Deciduoma Formation in the Hypophysectomized Pseudopregnant Rat

In the experiments of Evans *et al.* (1941) normal adult female rats were injected with the test preparations for 10 days beginning on the day of hypophysectomy. Threads were inserted in the uteri on the sixth day and the uteri examined for deciduomata on the eleventh. As the operation was not timed to coincide with that period of the œstrous cycle when corpora lutea capable of being sustained could be expected to exist, only some of the animals responded to potent extracts. If the pituitary were removed and the

injections begin within 24 hours of the first œstrous smear, this test would be more consistent.

A convenient, shorter, and more reliable method involves the induction of pseudopregnancy by electrical stimulation of the cervix uteri during œstrus. Five days later, the optimal time for endometrial traumatization, the hypophysis



is removed and injections begun immediately thereafter. The uterine trauma can be conveniently carried out by inserting a wire bearing a small barb at the tip through the cervix and up to the tubal end of one uterine horn. Active preparations give rise to an extensive decidual reaction in the damaged horn which can be detected as early as 24 hours after the stimulus, and which is conveniently evaluated at 72 hours. This method was used for studies of the chorionic luteotrophin of the rat (Astwood and Greep, 1938) and is equally applicable as a test for the hypophyseal factor.

The pregnant animal, hypophysectomized shortly after mating, can also serve as a test object; luteotrophic preparations maintain pregnancy for as long as they are given (Cutuly, 1942; Lyons, Simpson and Evans, 1943), and of course replacement therapy becomes unnecessary after the tenth to twelfth day of pregnancy when the "chorionic gonadotrophin" takes over (Astwood and Greep, 1938).

Structural and Chemical Changes in the Corpus Luteum

By the usual histological methods the persisting but non-functioning corpus luteum of the hypophysectomized rat is not appreciably different in appearance from one in which function has been maintained for a few days by luteotrophin. With prolonged treatment there is enlargement and altered staining characteristics of the cells, which later on become vacuolized. These changes are not sufficiently clear-cut to form a basis for an assay.

Everett has made a careful study of the structural changes in the corpus luteum of the rat during the œstrous cycle, during pregnancy, and after the injection of various hormones. The deposition of cholesterol in the corpus luteum, detected by the Schultz test on frozen sections, is apparently an early sign of regression. During the normal cycle cholesterol appears in small amounts early in diœstrus and slowly increases. When ovulation occurs in the next cycle a heavy deposition of cholesterol is seen. Luteinizing hormone is

thought to be the agent responsible for this change because it is seen 18 to 24 hours after the injection of luteinizing hormone preparations or of progesterone; the latter hormone is considered to evoke a premature release of luteinizing hormone from the pituitary if given at the proper stage of the cycle (Fig. 3). A single injection of œstrogen induces a similar sequence of events after a latent period of two days. Preparations of luteotrophin can reverse the cholesterol

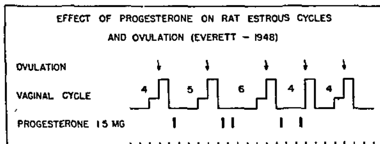


FIG. 3. Influence of progesterone upon the normal four-day cycle of the rat A single injection on the first day of dioestrus extended the cycle by one day. The expected five-day cycle [illegible]

deposition under these conditions, and in a personal communication Everett has suggested that an assay for this factor might be based upon this change. Apparently a single injection would be expected to cause a disappearance of the cholesterol in a matter of hours. Everett (1947) has shown too that a single injection of luteinizing hormone will induce regressive changes in the corpus luteum of pregnancy within 18 to 24 hours. This change and the associated increase in the cholesterol and ascorbic acid content of the corpus luteum can be prevented or reversed by the injection of luteotrophic extracts (Miller and Everett, 1948) Evidence was educed

(Everett, 1943) *that the corpora lutea of a preceding cycle* may resume function for a brief interval shortly before the succeeding œstrus. In *persistent-œstrus rats*, corpora lutea were induced by treatment with progesterone; a second cycle could then be completed with a single injection of progesterone, or with small doses of luteotrophic preparations.

The corpus luteum of the normal cyclic rat ceases to function before it is fully formed (Astwood, 1939) but, according to Everett, it releases progesterone some 18 to 24 hours before the next ovulation (presumably because it is stimulated by luteotrophin at this time). The progesterone acting upon the pituitary evokes a release of luteinizing hormone which, while inducing ovulation, also acts upon the old corpus luteum *to cause regressive changes*. After hypophysectomy, a previously functioning corpus luteum remains free of stainable cholesterol, but a single injection of luteinizing hormone will render it fatty and rich in cholesterol in 18 to 24 hours, a change which also is reversed by luteotrophic preparations given in a single dose. It should be possible to design a test comparable to the ascorbic acid depletion test for corticotrophin, which would be based on the cholesterol or ascorbic acid content of suitably prepared corpora lutea.

The recent finding of Saffran (1952) that corticotrophin can be tested directly *in vitro* with rat adrenal glands by measuring spectrophotometrically the cortical steroids produced during a 2-hour incubation period, suggests that luteotrophin might similarly be quantitated by measuring the amount of progesterone formed by luteinized ovaries in the presence of graded concentrations of the pituitary extract.

Summary

A review of the tests which have been used for the detection of luteotrophin showed that none is entirely satisfactory for assay purposes. Any one of several histological and chemical changes in the suitably prepared corpus luteum which can be detected within a few hours after a single dose of a luteotro- [illegible] preparation could serve as the basis for an assay method.

Even the direct measurement of progesterone synthesis *in vitro*, as influenced by luteotrophin, might eventually be used as an assay method.

REFERENCES

ASDELL, S. H., and HAMMOND, J. (1933) *Amer. J. Physiol.*, 103, 600.
ASTWOOD, E. B. (1939). *Amer. J. Physiol.*, 126, 162.
ASTWOOD, E. B. (1941). *Endocrinology*, 28, 309.
ASTWOOD, E. B., and GREEP, R. O. (1938). *Proc. Soc exp. Biol. Med*, 38, 713
ASTWOOD, E. B., RABEN, M S., and PAYNE, R W. (1952). *Recent Progr. Hormone Res*, 7, 1.
BRADBURY, J. T., BROWN, W E., and GRAY, L. A. (1950). *Recent Progr Hormone Res*, 5, 151
CUTULY, E. (1942) *Endocrinology*, 31, 13.
DRESEL, I. (1935) *Science*, 82, 173.
EVANS, H. M., SIMPSON, M. E, LYONS, W R., and TURPEINEN, K (1941). *Endocrinology*, 28, 933.
EVANS, H M., SIMPSON, M. E., and TURPEINEN, K. (1938) *Anat Rec.* (*Suppl* 3), 70, 26
EVERETT, J. W. (1943) *Endocrinology*, 32, 285
EVERETT, J. W. (1944) *Endocrinology*, 35, 507.
EVERETT, J. W. (1947). *Endocrinology*, 41, 364.
EVERETT, J. W. (1948) *Endocrinology*, 43, 389.
GREEP, R. O. (1938). *Endocrinology*, 23, 154.
GREEP, R. O (1941). *Anat Rec.*, 80, 465.
GREEP, R. O., VAN DYKE, H. B, and CHOW, B. F (1942). *Endocrinology*, 30, 635.
HAMMOND, J., JR., and ROBSON, J. M (1951). *Endocrinology*, 49, 384.
HECKEL, G. P., and ALLEN, W. M (1939). *Endocrinology*, 24, 137.
LAHR, E L., and RIDDLE, O. (1936). *Proc Soc. exp. Biol. Med*, 34, 880.
LOEB, L. (1923). *Proc. Soc. exp. Biol Med*, 20, 441.
LONG, J. A, and EVANS, H M (1922). Memoirs Univ. Calif., Vol. 6
LYONS, W. R. (1937) *Cold Spr. Harb Sym. quant Biol.*, 5, 198.
LYONS, W. R., SIMPSON, M. E., and EVANS, H M. (1943) *Proc Soc exp Biol. Med.*, 52, 134
MILLER, D C., and EVERETT, J W (1948) *Endocrinology*, 42, 421
N[illegible] *[illegible]ology*, 22, 86
N[illegible]
R[illegible] (1952) *Fed. Proc.*, 11, [illegible]
RABEN, M. S, WESTERMEYER, V. W., and LEAF, A (1952). *J. clin. Invest* (*Soc. Proc*), 31, 655
ROBSON, J. M. (1937). *J Physiol*, 90, 435.
ROSENBERG, I N. Personal communication.
SAFFRAN, M Personal communication

SAYERS, G., and ASTWOOD, E. B. Unpublished observations.
SMITH, P. E. (1930). *Amer. J. Anat.*, **45**, 205.
SYDNOR, K. L. (1945). *Endocrinology*, **36**, 88.
TEEL, H. M (1926). *Amer. J. Physiol.*, **79**, 170.
TOBIN, C. E. (1942). *Endocrinology*, **31**, 197.
WESTMAN, A., and JACOBSOHN, D. (1937). *Acta obst. gynec. scand*, **17**, 1.

DISCUSSION

FOLLEY: Dr. Astwood, may I ask if you advocate retention of the term "luteotrophin", as possibly denoting a different hormone from prolactin? Abraham White recently published a review on the physiology and chemistry of prolactin in which he adopted the term "luteotrophin". Would you say that was a permissible thing to do?

ASTWOOD One cannot explain all pituitary effects on the basis of the six well established anterior pituitary hormones. Until the situation has been more thoroughly studied I think one would be safer in referring [illegible]

may be.

GADDUM Do you agree to that?

FOLLEY. I think that is a conservative position which is perhaps desirable at the moment. Yes, I would agree.

ROBSON: I have recently tried to maintain the corpus luteum in the hypophysectomized rabbit with a preparation of prolactin (which I didn't make myself) and I haven't been able to convince myself that there has been any effect. That's a negative finding, which is unpublished and needs confirmation.

One thing startled me at the beginning of your communication—why did you restrict the use of luteotrophin to the non-pregnant animal?

ASTWOOD. Because I think that the chorionic gonadotrophins of mammals are also luteotrophins. It seems to me that the function of the chorionic hormone is to maintain the corpus luteum in pregnancy.

ROBSON: That can be shown experimentally, you mean?

ASTWOOD: Yes In man, chorionic gonadotrophin seems, as far as [illegible]

ROBSON. And you are entirely satisfied that the placental factor increases this period?

ASTWOOD: If one could forget about the rabbit. The rabbit is certainly an exception.

R. MORICARD You said that in pregnancy chorionic gonadotrophin maintains the function of the corpus luteum in woman?

ASTWOOD: That, I think, is its normal function.

R. MORICARD: Because it seems to me that it is quite a different

hypophysis

ASTWOOD In the human being during pregnancy there are tremendous quantities of chorionic gonadotrophin in the blood, as Dr Loraine has shown, and yet the ovary shows no signs of ovulation or of corpus luteum formation It shows, however, a functioning corpus luteum early in pregnancy. Furthermore, if one injects the urinary product into the normal non-pregnant woman, one can show a maintenance of corpus luteum function beyond the normal cycle. So I think the implication

SEGALOFF I object I think there is a lot of evidence that by administering adequate amounts of human urine chorionic gonadotrophin you get essentially the same effect on the ovary that you

corpus luteum) are exactly the same as you see in chorionepithelioma and in late pregnancy I feel that the effects in the human, given adequate dosage, are essentially the same as in pregnancy and that there really is very little evidence that the physiological activity of the material from the urine is any different from that in the serum.

LI May I ask Dr Astwood if there is any evidence that the so-called prolactin is dissociated from the luteotrophin. Have you any evidence that they are different?

ASTWOOD: No.

Li: Do you then intend to propose a new hormone when you use the term "luteotrophin"?

Astwood: No. As I pointed out, it may well be that they are the same, and it's widely held that they are, but I do not feel that the evidence is *final yet. As long as it isn't, one needs a test for this particular kind of* activity in order to be able to establish the question one way or the other.

Li: You might signify that by calling it "luteotrophin activity" test instead of "luteotrophin."

[illegible] That might be better.

Segaloff: If we are going to talk in general terms, and are going to consider man also, then I think that at least with the present evidence we have to consider that luteotrophin may be something else other than [illegible]

[illegible] perhaps already present. The second is that Freed and [illegible] her with [illegible] e latter [illegible] ring the *menstrual cycle.*

[illegible] other type of preparation

[illegible] this problem of what is [illegible] nt one, and I think it's

[illegible] ff, that by giving luteo- [illegible] extend the function of [illegible] under [illegible] appro- [illegible] is that [illegible] ything giving more prolactin [illegible] because the individual already has enough.

[illegible]

equivalent thing in man without redefining what we're going to do. And the prolactin won't do that in man.

LI: Perhaps you haven't found the right condition yet.

SEGALOFF: So far we haven't. That's why I say we haven't proved it But if the pituitary effects are identical we should be able to reproduce the rat experiment in man.

ASTWOOD: I consider that the luteal phase in the human cycle is homologous to the pseudopregnant phase of the rat or rabbit, and so the experiment is actually the same in the two species

R. MORICARD I think the question is quite different in man and the rat. You are speaking about immature female hypophysectomized rats and we are talking about normal human females, they are not comparable. We have studied one case of hypophysectomy in a woman; we have seen the degree of the vaginal, vulvar and uterine atrophy; and the effect of œstradiol is not the same as in castrated women. For me the comparison between women and the rat is difficult now.

P. MORRIS: I would like to ask Dr Astwood if he knows whether the so-called luteotrophin plays any physiological rôle in the human male?

ASTWOOD: No, I don't know of any function for it in man, nor of oxytocin.

P. MORRIS: Because Sonenberg labelled "prolactin" with radioactive iodine and traced the activity in the body. It's just a preliminary experiment, but he found the radioactive iodine in the prostatic gland

SOME OBSERVATIONS ON PROLACTIN ASSAYS BY THE PIGEON CROP-WEIGHT METHOD

PAMELA M. CLARKE and S. J. FOLLEY

THE results presented and discussed in this paper arise from a study of a series of assays of prolactin using the "systemic" pigeon crop-weight method. The objects of this investigation were to obtain further information on the accuracy of the assay method, in the light of changes in experimental material available, and to consider possible improvements in the technique.

Designs of the Assays

The statistical analysis was complicated by the fact that *the experimental design did not remain constant throughout* the 11 years under consideration, although generally a 4-point design, with two preparations each tested at two dose-levels, was used. Dose-levels varied from assay to assay, and because of supply difficulties so also did the ages and sometimes the breeds of the birds. Furthermore, since the assays were incidental to other studies, different seasons of the year were unequally represented. The basic experimental technique, however, which has been described by Folley, Dyer and Coward (1940), remained constant. Briefly, each bird received, on each of six successive days, a subcutaneous injection of the preparation dissolved in 1 ml. of aqueous medium, and was killed 24 hours after the sixth injection. An unheated pigeon house was used throughout, and the birds were fed *ad lib.* on wheat and water.

In the majority of the experiments the birds were arranged in blocks on the basis of body weight at the start of the assay (*initial* body weight), but a cyclic rather than a random arrangement was then used to allocate the birds to treatment

groups. Although the analysis showed that adjustment for this non-random arrangement scarcely affected the estimated variances and differences between mean responses, the procedure is not recommended, and assays are now based on orthodox statistical designs.

The birds were usually kept three in a cage, all birds within a cage receiving the same treatment, and in general they were allocated to cages in descending order of body weight.

Method of Analysis

In previous work, various response metameters, such as crop weight alone and crop weight as a percentage of initial body weight or body weight at autopsy (final body weight), have been used. Here, however, the crop weight has been used for analysis, with covariance analyses on initial body weight as an auxiliary variable Adjustment was made where necessary to allow for the cyclic system of allocation of birds to treatments, and where appropriate, effects attributable to differences between breeds of birds in the same experiment were eliminated from the various comparisons considered. Since analyses of the results of assays in which there was no blocking did not show a greater variation in crop weight between cages than that within cages, differences between cages were not eliminated in the analyses.

Changes in Response with Time

The assays studied were carried out during the years 1940–1950, and over the first five years of this period the standard preparation used was the International Standard prolactin From 1944 onwards, a sheep pituitary preparation from Schering Inc., with a potency which we have estimated to be 21·8 i.u./mg. (5 per cent fiducial limits 19·0 and 24 9 i u./mg.) was used as a laboratory sub-standard.

Fig. 1 shows the mean responses to a total dose of 1·2 mg. (12 i u.) of the International Standard preparation, obtained by direct measurement or by interpolation at different times, and Fig. 2 shows the corresponding mean slopes of the log

dose-response curves for total doses from 6 i.u. to 24 i.u. Both variables have been plotted against the dates when the assays were performed. It can be seen that the mean response increased with time, from 2·39 g. crop weight in 1940 to 4·80 g. crop weight in 1944, and the mean slope also showed

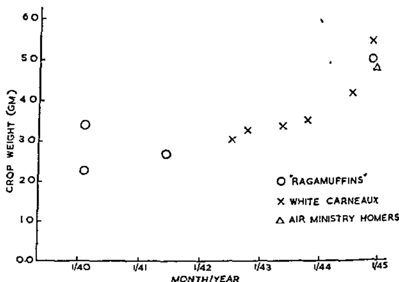


FIG. 1. Mean crop-weight response to a total dose of 1 2 mg of International Standard prolactin.

a possible increase with time, although this was not so well defined.

Various explanations for a progressive increase in response may be considered. Some part of the response differences can perhaps be attributed to breed: as shown on the graph, in four of the experiments the birds were dealers' "ragamuffin" pigeons, of mixed breeds and of unknown ages; in one experiment the pigeons were Air Ministry homers, of uniform appearance; for the remainder of the assays White Carneau pigeons from the A.R.C. field station at Compton were used.

To examine possible association of crop-weight response with the passage of time as well as with initial body weight

and gonad size, partial regression coefficients were calculated. In this analysis, adjustments were made for breed and seasonal differences, and since the mean crop weights were estimated with different precisions, they were weighted inversely as their variances. The coefficient of partial regression of crop

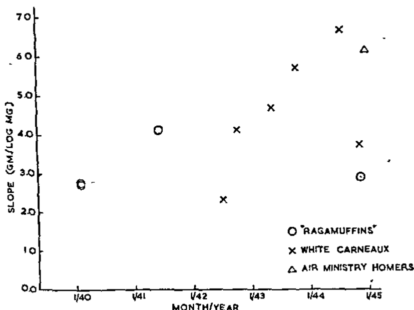


FIG 2. Slope of mean response to International Standard prolactin

weight on time was highly significant ($P < 0 \cdot 01$), but no other source of variation examined was significantly related to the crop weight. Similar results were obtained when the mean crop weights for the White Carneau birds only were analysed. For the slopes of the log dose-response curves, the coefficient of partial regression on time was significant at the 5 per cent level. It therefore appears reasonably likely that both changes with time are real.

Attempts to attribute these [illegible] sions [illegible]

We [illegible] same period on any other spe[illegible] response to this preparation, or on crop weight responses to any other prolactin preparation. Although it is possible that the activity of the International Standard preparation may have undergone a progressive change (and any such change may or may not have been specific to this preparation, or to these stor[illegible] lar type of response), thi[illegible] sically unlikely. Condi[illegible] management of the birds may, of course, have altered in some unnoted way, or the birds may have become more sensitive to prolactin. It may be noted that Hall (1944) reported a decreased response over a period of two years to the same dose of a prolactin preparation, using birds of uniform breed and age, from the same source throughout. Moreover, an increase with time in the thyroid weights of the same strain of pigeons has been reported (Bates, Riddle and Lahr, 1941), and some such physiological change may be responsible for the changes in response noted here.

The corresponding results for the laboratory sub-standard preparation over the years 1944 to 1950, illustrated in Figs. 3 and 4, do not show any obvious trends with time, and using a similar analysis no significant partial regression coefficients were found.

Neither the results for the International Standard prepara-tion nor for the laboratory sub-stand[illegible] semi[illegible] by [illegible] found mini-mu[illegible] October-November and in April-May. The representation of months here, however, is so uneven as to make our evidence on this point inconclusive. It appears unlikely, from examination of the dates of the assays, that such a cyclic effect could account for the apparent increase with time of the response to the International Standard preparation.

FIG 3 Mean crop weight response to a total dose of 0 6 mg Schering sheep prolactin

FIG 4 Slope of mean response to Schering sheep prolactin.

Attempts to attribute these increases in mean response and slope to any particular cause or causes can only be speculative. We have no evidence over the same period on any other specific response to this preparation, or on crop weight responses to any other prolactin preparation. Although it is possible that the activity of the International Standard preparation may have undergone a progressive change (and any such change may or may not have been specific to this preparation, or to these storage conditions, or to this particular type of response), this explanation is thought to be intrinsically unlikely. Conditions of management of the birds may, of course, have altered in some unnoted way, or the birds may have become more sensitive to prolactin. It may be noted that Hall (1944) reported a decreased response over a period of two years to the same dose of a prolactin preparation, using birds of uniform breed and age, from the same source throughout. Moreover, an increase with time in the thyroid weights of the same strain of pigeons has been reported (Bates, Riddle and Lahr, 1941), and some such physiological change may be responsible for the changes in response noted here.

The corresponding results for the laboratory sub-standard preparation over the years 1944 to 1950, illustrated in Figs. 3 and 4, do not show any obvious trends with time, and using a similar analysis no significant partial regression coefficients were found.

Neither the results for the International Standard preparation nor for the laboratory sub-standard prolactin show a clear semi-annual cycle of the crop-weight response, such as reported by Bates and Riddle (1941) and Hall (1944), who found minimum responses in October-November and in April-May. The representation of months here, however, is so uneven as to make our evidence on this point inconclusive. It appears unlikely, from examination of the dates of the assays, that such a cyclic effect could account for the apparent increase with time of the response to the International Standard preparation.

Comparisons of Crop-Weight Responses for Different Breeds

In each of three experiments there were sufficient birds of different breeds to permit useful comparisons of the variation and sensitivity to prolactin for different breeds or types. The results of these comparisons are summarized in Table I.

Table I

RESULTS FOR DIFFERENT BREEDS OF PIGEON

Date of first injection	7 5 42	2 11 44	23 7 48
Mean initial weights (g)			
"Ragamuffins"	305±12 1	345± 8 7	—
White Carneaux	454±11 2	490±11 6	458±6 6
Silver Kings	436±13 3	—	445±7 6
Mean crop weights (g)			
"Ragamuffins"	3 41±0 328	5 54±0 478	—
White Carneaux	2 99±0 178	5 86±0 221	3 36±0 169
Silver Kings	4 01±0 239	—	2 99±0 252
Mean slopes (g /log mg)			
"Ragamuffins"	—	3 39±1 743	—
White Carneaux	—	5 06±0 810	5 73±0 545
Silver Kings	—	—	6 49±0 918
Estimated error variances			
"Ragamuffins"	1 396(11d f)	3 659(8d f)	—
White Carneaux	0 477(13d f)	1 970(24d f.)	0 716(20d f)
Silver Kings	0 860(13d f)	—	1 018(8d f.)

The "ragamuffin" birds in each experiment had a lower mean initial body weight than the Silver Kings and White Carneaux ($P<0\ 05$); for all three types of bird the variations of the initial weights about their mean values were similar.

There were no consistent significant differences between crop-weight responses for birds of different breeds. Division

Consistency of the Assay

Although considerable variation between assays was observed, both in the crop-weight response to the same dose of prolactin and in the slope of the log dose-response curve, assays of the same test preparation at different times were not, within the limits of experimental error, in disagreement. Data for examination of the repeatability of the assay were, however, available only for two preparations.

Between October, 1943, and December, 1944, four assays of the Schering sheep prolactin relative to the International Standard were made The breeds, ages and numbers of birds available varied during this period, and in one assay only one dose-level of the sheep prolactin was used. The variances of the four estimates of relative potency showed significant deviations from homogeneity ($P<0{\cdot}001$). In chronological order, the relative potency estimates were 20·7, 31·0, 23·0 and 21·4 I.U per mg., but the variance of the outstandingly high estimate was also relatively high, and any departure of the four values from homogeneity was not significant at the 5 per cent level. To obtain the mean estimate of relative potency and its fiducial limits quoted earlier, the four separate estimates were weighted inversely as their variances, and the fiducial limits were calculated by the method, due to Cochran, described by Bliss (1951). It is perhaps worth noting that in the assay giving the least well determined estimate of relative potency, birds believed to be more than two years old were used, whereas the birds in the other experiments were either known or believed to be less than one year old

On two occasions, separated by an interval of 15 months, another prolactin preparation was assayed against the laboratory sub-standard. Here also the birds used in different assays were of different breeds, and perhaps of different ages. The difference between the two estimates, 0·47 and 0·53, of relative potency was not significant ($P>0{\cdot}05$).

scoring system—0 for small, 1 for medium and 2 for large—have been plotted against ages of birds.

Examination of the most recent experiments, all involving pure-bred birds from the same source, revealed a strong correlation between gonad size and assay precision. Table II summarizes results for the Schering sheep prolactin in the last

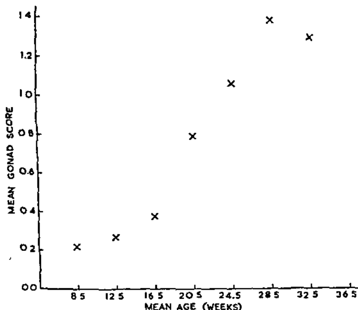


FIG. 5. Relationship between mean gonad score and age

seven experiments, carried out over a period of four years. The birds used were White Carneau pigeons from Compton field station, except that in the last experiment there were 7 Silver Kings and 13 White Carneaux. The table shows, for each assay: the approximate range, where known, of ages of the birds; the mean initial weight; the mean gonad score, obtained as described above; *s*, the standard error per bird; and *b*, the mean slope of the log dose-response line. To give an index of the precision of each assay, the quantities *s*/*b*,

by the initial body weight thus introduces differences between breeds: in terms of crop weight per 100 g. body weight the mean response for "ragamuffins" was higher in each experiment than the corresponding value for White Carneaux or Silver Kings ($P<0 \cdot 05$).

There were indications that the responses of the "ragamuffin" pigeons were more variable than those of the White Carneaux and that the "ragamuffins" were less sensitive to increased doses of prolactin, but these differences were not found to be statistically significant.

Relationship between Gonad Size and Crop-Weight Response

For each bird, in every experiment, a record was made of the gonad size at autopsy, the gonads being classified by inspection as small, medium or large. Analyses were made where possible to examine the relationship between gonad size and age, sex, breed, prolactin treatment and crop-weight response to the *same treatment*.

Within any one experiment gonad size as here recorded showed no significant correlation with the crop-weight response χ^2 tests within the experiments for which ages of individual birds were recorded showed a highly significant relationship between age and gonad size, as classified. Sex, breed and treatment differences did not appear to affect this relationship, nor was there any evidence of a significant treatment effect on gonad size. Since these comparisons *were made within assays, seasonal differences were eliminated.* The experiments were too few to give any conclusive evidence on effects of seasonal changes on the relationship between gonad size and age, but no significant differences in this relationship were found between assays, nor was there any significant difference between the form of association of age and gonad size obtained by comparison of different assays and that obtained by within-assay comparisons.

The relationship between gonad size and age is illustrated in Fig. 5, where mean gonad ratings based on an arbitrary

mum precision was achieved with young pigeons not more than three months old. This finding gives a foundation to the practice, recommended by Riddle, Bates and Dykshorn (1933), of using birds between 6 and 10 weeks after hatching. In this connection, Hall (1944) advised against the use of very young pigeons, since the crop-sacs of pigeons 2–3 weeks after hatching were found by Bates, Riddle, Lahr and Schooley (1937) to show signs of proliferation, not visible in pigeons six weeks old.

Precision of the Assay

Folley *et al.* (1940), in a statistical analysis of earlier assays, calculated the limits of error, at three probability levels, that would be expected using various numbers of birds. At that time, however, only birds of mixed breeds and unknown ages were available, and now that a source of supply of inbred White Carneau pigeons, of known age, has been established, a further estimate of the number of birds required for satisfactory limits of error was of interest. As just shown, however, the value of λ for the most recent assays varied considerably, probably due to age differences. On the assumption, based on the results shown in the graphs, that the use of young birds 2–3 months old will give a value of λ approximately equal to 0·11, the estimated numbers of birds required for given fiducial limits of error are, surprisingly, no smaller than those reported previously. For a 4-point assay, it is estimated that 10 birds on each treatment would give 5 per cent fiducial limits of 76 per cent to 131 per cent, 15 birds per treatment would give limits of 88 per cent to 122 per cent, and 20 birds per treatment would give limits of 85 per cent to 118 per cent. These numbers of birds may, however, be over-estimates, since a lower value of λ might result if the range of ages within an experiment were smaller than obtained here.

Incidental Points

Among incidental points arising from the analysis, there was an indication that the increase in body weight during the

denoted λ by Gaddum, have been calculated. For a constant number of birds, the precision increases as λ decreases.

Table II

RESULTS OF ASSAYS OF LABORATORY SUB-STANDARD PROLACTIN

Date of first injection	*Ages of birds (months)*	*Mean initial weight (g)*	*Mean gonad score*	*s (g)*	*b (g. per log mg)*	λ
17 9 46	3–7	523	0 60	0 510	3·82	0 134
8 10 46	3–8	514	0 35	0 756	6·14	0 123
28 8 47	2½–3½	494	0 08	1 001	9 50	0·105
23 7 48	5–8	452	1 38	0 657	4 42	0 149
24 2 49	?	537	2 00	1 035	5 62	0 184
25.8 49	4½–6	451	0·58	1·025	7 46	0·137
13 7.50	13–15	506	1 75	1 188	5 99	0 198

No obvious connection between b or s and the time, age, gonad score or initial body weight was found. There was, however, a clear relationship between the mean gonad score and λ, showing, as illustrated in Figs. 5 and 6, that the maxi-

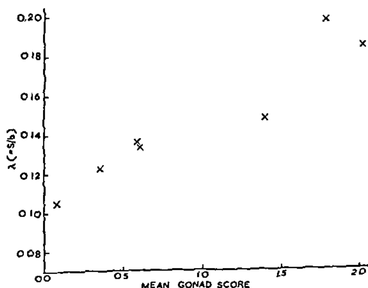


FIG. 6. Relationship between mean gonad score and assay precision.

in blocks according to initial body weight has, under our conditions, in most cases usefully increased the precision of the assay. If there is no blocking on a body weight basis, adjustment for initial body weight may give increased precision, and it is considered that to this end crop weight expressed as a percentage of the initial body weight may be as effective a metameter as the crop weight adjusted for initial body weight on the basis of an analysis of covariance. It is possible also that the precision of the assays might be improved by the use of individual cages, thus permitting more efficient elimination of positional differences; but the assays studied here give no information on this point. Smaller doses of prolactin might also prove advantageous, if the anticipated reduction in error variation were more than sufficient to compensate for the decrease in the slope of the log dose-response curve

We are indebted to Dr. Erwin Schwenk, then of the Schering Corporation, Bloomfield, N J , for the gift of the sheep prolactin preparation used as a laboratory sub-standard, to Dr W S Gordon, Director of the Agricultural Research Council's Field Station, Compton, Berkshire, and his staff for valuable co-operation in the supply of pigeons, to Mr C. P. Cox for useful discussions, and to Mr S C Watson for skilled technical assistance in the assays

REFERENCES

BATES, R. W , and RIDDLE, O (1941) *Endocrinology*, 29, 702
BATES, R. W , RIDDLE, O., and LAHR, E L. (1941). *Endocrinology*, 29, 492
BATES, R W , RIDDLE, O., LAHR, E. L., and SCHOOLEY, J. P. (1937). *Amer. J. Physiol* , 119, 603
BLISS, C I (1951). Vitamin Methods, ed Gyorgy, P New York. Academic Press, Inc
FOLLEY, S. J , DYER, F. J., and COWARD, K H. (1940) *J Endocrinol.*, 2, 179
HALL, S R (1944) *Endocrinology*, 34, 14.
RIDDLE, O , BATES, R W , and DYKSHORN, S. W. (1933). *Amer. J. Physiol* , 105, 191

DISCUSSION

ASTWOOD. Have you any information on the effects of subcutaneous versus intramuscular injection, divided doses versus one injection per day, and the influence of the pH of the solution in which the prolactin is dissolved?

assay was greater for higher doses of prolactin given, so that it is considered preferable to adjust crop weights for initial rather than final body weights. After adjustment for initial body weight no significant differences in response were found between the sexes, a result in agreement with that of Hall (1944). There were indications that the variation in crop weights was less for birds caged in twos than for birds caged in threes, but when tested this was not found to be statistically significant.

Discussion

The quantitative and objective nature of the observations involved in the pigeon crop-weight method of assaying prolactin are points in its favour, although the large number of birds estimated to be necessary for satisfactory precision, at least under the conditions obtaining in our laboratory, is a drawback, particularly since pure-bred pigeons are at present expensive in this country. Some workers have described methods based on subjective judgements of the degree of stimulation of the crop-sac, but information on the precision of assays of this type is not readily available, and comparisons of the repeatability, precision and economy of material for the different assay methods are therefore impossible. "Micro" methods using intradermal injections over the crop-sacs have proved useful in assaying small quantities of prolactin and also as a qualitative test, and have the additional advantage of giving quicker results. For most purposes it would perhaps be preferable to have an assay method based on lactation responses in mammals, but as yet we are not aware of any mammalian assay method sufficiently sensitive for general purposes.

On the basis of the analysis of the series of assays considered here, in which subcutaneous injections on each of six successive days were made, the use of young birds of no more than three months old, and of uniform breed, with concurrent tests at two or more dose-levels of both the standard and the test preparation are recommended. The arrangement of the birds

FOLLEY: I have very little experience of that method, but I always felt that the end-point is rather subjective, and that different people might give different opinions as to whether or not minimal stimulation had occurred. I felt that for our purpose, where we wanted to assay preparations as accurately as possible, that the crop weight method was really the most objective and less open to individual variations between observers.

SEGALOFF: I agree with Dr. Folley. We tried minimum stimulation having more than one person do the estimations and having no idea what the birds got, and when we got through we had no idea what the birds got either!

GADDUM: Do you feel that this is the most accurate method?

FOLLEY: Yes, definitely. I think the minimal method is very useful for controlling chemical purification of prolactin, as a sort of semi-quantitative test, but personally I feel that for real accuracy in assaying a preparation, this is the best that is available

GADDUM: One of your lambdas was as good as the one that Dr. Segaloff produced.

for a constant dose, the mean response to subcutaneous injections was higher than the mean response to intramuscular injections. I think I'm right in saying that the volume of the injectorate has also been found to affect the response.

FOLLEY: We always adjust the *pH* as near neutrality as possible to keep the hormone in solution I would say that in all these assays you can take the *pH* as being never higher than *pH* 8.

LI Perhaps I could answer Dr Astwood's question about the comparison of subcutaneous and intramuscular administration. The subcutaneous method is about twice as sensitive as the intramuscular, in our experience: the minimum effective dose subcutaneously is 0 1 mg. per day; intramuscularly, about 0 2. The *pH* did not affect the sensitivity very much, we changed it from *pH* 4 up to *pH* 8 We thought at one

times Copper sulphate and zinc sulphate have been tried, but they have not increased the activity.

decreases very markedly after it comes into the laboratory—sometimes as much as 100 g. So we always take the final body weight of our assay animals

CLARK: We also generally experienced a loss in the body weight of the pigeons This loss in weight was in most cases greater for birds receiving low doses of prolactin than for those receiving higher doses, and it was this indication of a relationship between size of dose and loss in body weight that led us to use the initial rather than the final

attempts to utilize the local crop
. Fig. 1 illustrates why this was
reaction, which resembles fat
the crop response but, as well as
with absorption of the hormonally
site of injection. When attempts
r such extracts subcutaneously or
swelling of the area, which had the
ould actually develop in the animal.
ts was it possible to concentrate the
ng with it this non-specific reacting
was necessary to substitute for the
ction the extraction procedure and
utlined.

tration involves alcohol precipitation,
the precipitate and reprecipitation.
lly prepared, relatively non-toxic to
ccasional specimen will kill one or

of Prolactin from Urine
rine specimen, refrigerated during collection

ght of NaCl
acetic acid if necessary
cent alcohol
ght in refrigerator
decantation, siphoning off supernatant, and

*per cent alcohol, twice with ether; dry over-
mperature*

tate with 3×15 ml. dist. H_2O, 30 min with

rly changes of 0 5 per cent NaCl at 5–8°C

ct within membrane add 0 1 g NaCl (as 0·5
ution) and 4 volumes of 95 per cent alcohol

overnight in refrigerator.
precipitate as before.
r assay.



FIG. 1. Non-specific reaction from local injection of urinary prolactin concentrates.

To face page 107]

Table I

CALCULATION of RESULTS of ASSAY

y = average $\frac{\text{crop weight}}{\text{body weight}}$ for each group of 4 birds

It is assumed that the ratio of y varies linearly between 2.5 IU and 40 IU + 2.5 IU

This implies $\frac{u}{y - y_{2.5}} = \frac{40}{y_{40} - y_{2.5}}$

where u = units of urinary prolactin received by each bird.

Hence $u = \left(\frac{40}{y_{40} - y_{2.5}}\right) \left[y - y_{2.5}\right]$

and $IU = 5\left(\frac{40}{y_{40} - y_{2.5}}\right) \left[y - y_{2.5}\right]$

Let $C = 5\left(\frac{40}{y_{40} - y_{2.5}}\right) = \frac{200}{y_{40} - y_{2.5}}$

calculated from control data for each assay group (10 patients) $IU = C\,(y - y_{2.5})$

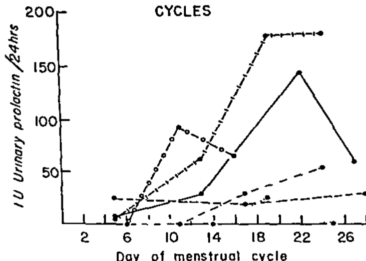


FIG. 2 Prolactin excretion in normal menstrual cycles.

Assay Procedure

1. *Preparation of Extract for Injection*

   Residue ground and extracted with 40 ml. H_2O dist. for 2 hr. After centrifuging, the supernatant is decanted

2. *Injection*

3. *Controls*

   (a) Group given only the 2·5 i.u. subcutaneous dose
   (b) Group given i.v. 40 i.u. per pigeon + 2·5 i.u. subcut.
   (c) Group uninjected.

4. *Autopsy*

   5th day—birds killed and weighed. Crop removed, spread flat, cleaned, opened, blotted dry and weighed at once.

There is one major difference in this assay procedure as opposed to that presented previously by us (Coppedge and Segaloff, 1951) in that instead of adding the booster hypophyseal prolactin to the material administered intravenously, it is given as a separate subcutaneous injection, with two purposes in mind. First, it saves hypophyseal prolactin, and secondly, it obviates the question that the positive assays are due to leakage of the much larger amount of intravenously administered hypophyseal prolactin around the site of injection.

In Table I is a brief run-down of the method of assay calculation. This is simply a reduction to mathematical terms of the assay results so as to avoid plotting each assay.

Only a few results which are suggestive of the usefulness of this assay procedure will be presented. Fig. 2 is a composite of the curves obtained from several normal young women on whom more than two specimens could be collected during the cycle. With one exception, all these curves show a rise at the latter half of the cycle. This suggests that the prolactin-like material being measured may be indeed luteotrophin.

Acknowledgments

These studies have been made possible by grants from the Committee on Endocrinology of the National Research Council; the National Cancer Institute of the National Institutes of Health; Ayerst, McKenna and Harrison, Inc.; and The American Cancer Society.

We would like to thank Drs. R. L. Coppedge and M. Duren, Mr A. Flores, Mrs. R. L Coppedge and Miss R. Cleo Bell for their co-operation in carrying out these studies.

REFERENCES

COPPEDGE, R. L , and SEGALOFF, A (1951) *J. clin. Endocrinol* , **11**, 465.

SEGALOFF, A., GORDON, D , HORWITT, B. N., SCHLOSSER, J. V , and MURISON, P. J. (1951). *Cancer*, **4**, 319.

DISCUSSION

FOLLEY: In your assay, how many doses did you give of each preparation?

SEGALOFF· One dose of urinary extract. Four birds for the unknown. Four birds for two levels of laboratory standard.

FOLLEY Twelve altogether?

SEGALOFF· Yes But actually several urines

FOLLEY: Were they all given the booster dose?

SEGALOFF· In the past in each assay we have included some without the booster dose, but it's too expensive in terms of birds and other costs In the actual assay now we always use the booster dose, and have stopped carrying the laboratory standard in birds that don't get the booster dose. So actually we give all the birds the booster dose subcutaneously and then the two levels of the laboratory standard intravenously.

FOLLEY Is that a Bates prolactin?

SEGALOFF. Yes, a Bates sheep prolactin

FOLLEY. About 22 i u /mg ?

SEGALOFF. It's almost exactly 20.

CROOKE In view of the very severe local reaction, have you tried using inunction at all?

SEGALOFF: Yes, and it doesn't work. Maybe we just haven't put the material in the right vehicle

HARRIS· Is there any data as to what part the animal's own pituitary

jected œstrogen, or whether it might have been due to "suppression" of the animal's pituitary, as it might possibly have been

Fig. 3 shows the data obtained during a few pregnancies. Unfortunately most of these are scattered values, but here too there seems to be a tendency toward increase as pregnancy progressed.

There are also small increases in prolactin excretion when patients are treated with luteotrophic doses of chorionic

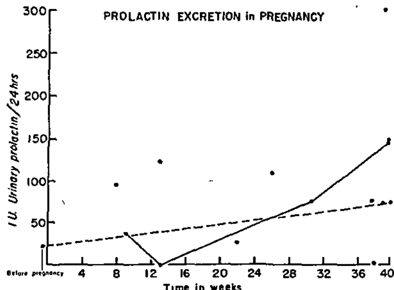


FIG 3. Prolactin excretion in pregnancy.

gonadotrophin, which would seem to fall in line with these values presented for pregnancy.

Prolactin excretions have also been followed in a fair number of patients with advanced cancer of the breast who were being treated with various types of steroids; and to date the only steroid that seems to produce a consistent change is testosterone propionate, which produces an increase (Segaloff, *et al.*, 1951).

In summary, we have presented a method for the assay of urinary prolactin activity and the results obtained in applying this assay in some representative normal and pregnant women.

and Harrison, Inc.; and The American Cancer Society.
We would like to thank Drs. R L. Coppedge and M. Duren, Mr. A. Flores, Mrs. R. L. Coppedge and Miss R. Cleo Bell for their co-operation in carrying out these studies.

REFERENCES

COPPEDGE, R. L., and SEGALOFF, A (1951) *J. clin Endocrinol.*, **11**, 465.

SEGALOFF, A., GORDON, D., HORWITT, B N, SCHLOSSER, J. V, and MURISON, P. J. (1951). *Cancer*, **4**, 319.

DISCUSSION

FOLLEY: In your assay, how many doses did you give of each preparation?

SEGALOFF: One dose of urinary extract. Four birds for the unknown, Four birds for two levels of laboratory standard

FOLLEY: Twelve altogether?

SEGALOFF: Yes But actually several urines

FOLLEY. Were they all given the booster dose?

SEGALOFF: In the past in each assay we have included some without the booster dose, but it's too expensive in terms of birds and other costs. In the actual assay now we always use the booster dose, and have stopped carrying the laboratory standard in birds that don't get the booster dose So actually we give all the birds the booster dose subcutaneously and then the two levels of the laboratory standard intravenously.

FOLLEY: Is that a Bates prolactin?

SEGALOFF: Yes, a Bates sheep prolactin.

FOLLEY: About 22 i.u /mg ?

SEGALOFF. It's almost exactly 20

CROOKE: In view of the very severe local reaction, have you tried

if you give œstrogen to pigeons at the same time as prolactin, again you get a decreased response. We never went into that further to find out whether the decreased response was due to anorexia caused by the injected œstrogen, or whether it might have been due to "suppression" of the animal's pituitary, as it might possibly have been

ASTWOOD: If the substance being tested is the same as the material given as the booster dose, I can't see how greater sensitivity or greater precision can be obtained I should think it would imply that the material given was qualitatively different.

SEGALOFF: I don't see why it has to. In our experience, and also from the published and unpublished data of both Bates and Hall, you have to give a fairly substantial amount before you start getting increases. The 2 5 unit equivalent that we use as a booster dose will give a barely detectable response, and adding another increment of 2·5 units to that gives you a tremendous response.

GADDUM The dose effect curve must be S-shaped, and you have to get on the steep part of it.

SEGALOFF Yes, that's it.

FOLLEY: We have actually found it to be S-shaped.

of

h-
he

ROBSON· In the sites where you injected the preparations and got a local reaction, did you get leucocytic infiltration?

SEGALOFF. There is a fair amount.

ROBSON: We've injected gonadotrophin locally into corpora lutea, and you get a very curious type of reaction there with cellular infiltration, and the formation of layers of fibrous tissue around it. And that's a reasonably pure gonadotrophin. I wondered whether there was any relation between these effects, and whether you might possibly eliminate your local reaction if you could eliminate the gonadotrophin from the prolactin extract

n
u

get a special type of urinary prolactin A foreign body reaction of that *type has been demonstrated with preparations of complex polypeptides*

SEGALOFF: Of course any untreated urine we've ever tried will do this.

CLARKE: I should like to ask Dr. Segaloff if it has been possible for him to obtain any evidence on the similarity of the log dose-response curves for the urine preparations and the international standard

SEGALOFF· That's one of my troubles. I haven't been able to build up a big enough supply of material to get good enough data to talk about the comparison In the few experiments we have done, mostly using the preparations made from pregnancy urine, it does look as though they're parallel curves

MACLAGAN
about the re
surprising. Y

treatment with testosterone, which was rather the reverse of what I would have expected.

SEGALOFF: Why the testosterone did it, I don't know. We used many other steroids, and so far testosterone is the only one that shows an effect. It is not 100 per cent consistent—although the decrease of gonadotrophin with testosterone administration is very consistent (it occurred in all the [illegible]

MACLAGAN: Did the results of the assay have any relation to the clinical response to treatment?

SEGALOFF: We have been unable to correlate the prolactin results with [illegible]

have obtained in normals, there is a definite tendency to increase with age. Our highest values have been obtained in the few we've had in [illegible]

who show a lower than expected gonadotrophin value have practically all fallen in the group who have failed to respond to testosterone. The same is true of the other steroids, but we don't have as much data as [illegible]

that cancer is a chronic illness?

ROBSON: Have you done any male urines?

SEGALOFF: Yes, a great many. And the activity occurs in male urine. I don't know what it's doing there. As in women, the highest values have come with the oldest ones, and in the few boys that we've done we've found nothing.

ROBSON: How do the activities in the male compare with the female values? Do they come up to the high or low level of the menstrual cycle?

SEGALOFF: They're mostly at the lower level of the menstrual cycle.

ROBSON: Do you get higher ones too?

SEGALOFF: Only in the few old men we've tried.

GILLILAND: Have you tried any men with Klinefelter's syndrome?

SEGALOFF: We have, but at the moment I don't remember what we got.

FOLLEY: Have you tried any hypogalactia cases, as compared with normal post-parturient women?

SEGALOFF: We have a few, and the values seem to be about the same. We've had several cases of persistent lactation, both with and without amenorrhœa, and we have two of them in whom we have followed the prolactins all the way through the cycle, and in both of them the values are distinctly higher than most of the values obtained in the others. I might add, however, that the commonest way to cure this peculiar syndrome is to refer the patients to a psychiatrist; in both of these patients the persistent lactation ceased afterwards.

FOLLEY: Would you say that psychiatrists could cure the Chiari-Frommel syndrome?

SEGALOFF: I would hesitate very much to say so. I hate to tag a name like that on to what I think is an exceedingly heterogeneous group of patients.

LORAINE: Have you any observations in pathological conditions during pregnancy?

SEGALOFF. One of our patients developed a toxæmia in the midst of [illegible] We had a specimen collected just

care of her to ask for another specimen, we discovered that she was in the hospital with pre-eclampsia. She lost the baby that time. She became pregnant again and developed clinical diabetes at the sixth month of pregnancy. We haven't completed all the assays, but her pregnanediol excretions just before the diabetes came on were lower than they had been before. That's the one accident of pregnancy that we've had so far.

FINGL. When you estimate that a 24-hour urine sample contains 100 i.u. per 24 hours, what is a good estimate of the error of that determination?

SEGALOFF: I honestly don't know what a *good* estimate of it would be.

FINGL: Could the estimate be off as much as two-fold?

SEGALOFF: Using four birds as we do, and if the results are quite consistent, I don't think it's very likely. We have several people, including some of the men in the laboratory, from whom we have as many as 5 and 6 determinations, in which the greatest error is about 4 or 5 per cent in the determinations, yet we have others in which there has been a much bigger spread. But some of our breast cancer patients, for example, on whom we have several determinations in periods during which they're getting no therapy, have been quite consistent, and I would say that most of them have not varied by more than about 20 per cent.

BIOASSAY OF GROWTH HORMONE*

C. H. LI

Introduction

METHODS which have proved useful for the bioassay of growth hormone will be briefly outlined and discussed here. An evaluation of these methods by direct comparison is difficult, since they have been standardized in different laboratories, under different conditions, and with hormone preparations of different potency and purity. However, certain of the methods have been studied in this laboratory using the same hormone preparation, and from these data some evaluation of the procedures can be attempted. A list of the methods, given below, includes both those which have been thoroughly studied and are generally accepted as assay procedures, and those which have been suggested for the bioassay of growth hormone but which either have not gained general acceptance or have been insufficiently explored.

Methods Available for the Bioassay of Growth Hormone

A. Well-established Procedures.

1. *Increase in weight of normal plateaued rats.*
2. Increase in weight of hypophysectomized rats.
3. Increase in weight of dwarf mice.
4. Increase in tail length of hypophysectomized rats
5. Increase in width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats.

B. Suggested Procedures

1. Increase in liver weight
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]
6. [illegible] adioactive tracers

*Aided in part by research grants from the American Cancer Society, the Albert and Mary Lasker Foundation, and the Rockefeller Foundation.

Increase in Weight of Normal Plateaued Rats

One of the first noted and most easily demonstrable effects of growth hormone is the acceleration of the growth of normal rats. The details of the procedure as used in this laboratory may be briefly outlined as follows:—

Adult female rats of the Long-Evans strain, 5 to 6 months old, weighing 220 to 280 g., in which growth stasis can be demonstrated by failure to gain more than 10 g. in 20 days, *are satisfactory for the assay procedure. Male animals have* not been as satisfactory as females. Diet and animal room conditions should be kept constant. The animals are weighed at 5-day intervals. Injections of hormone are made intraperitoneally daily for a specified period of time. Originally a 20-day test period was used, but a 15-day injection period has been found to be adequate. Growth hormone preparations of different potency tested by this method have produced log dose-response curves which are very nearly parallel, indicating that the response is reproducible. There is some variation in the responsiveness of the animals at different times of the year, so that it is essential to control assays with a standard hormone preparation, preferably the pure hormone, whenever possible

Increase in Weight of Hypophysectomized Rats

Cessation of growth in the hypophysectomized rat and its resumption following implantation of whole pituitary glands was first demonstrated by Smith (1926, 1927, 1930). Among the first to utilize these phenomena in the assay of growth-promoting extracts of the anterior hypophysis were Van Dyke and Wallen-Lawrence (1930). The procedure for the hypophysectomized rat weight test as used in this laboratory may be described as follows:—

Immature female rats are hypophysectomized at 26 to 28 days of age, and, if evidence for completeness of operation has been obtained, are used for assay 10 to 12 days later. The criteria for completeness of hypophysectomy are: limitation of body weight gain to 7 g. in the pre-injection period,

impairment of body tonus, maintenance of infantile hair, and the condition of the sella turcica at autopsy. The hormone is injected intraperitoneally daily for 15 days, or, more recently, for 10 days.

When it was analysed statistically it was found that the hypophysectomized rat weight test is slightly less precise than the plateaued rat weight test. This is substantiated by the fact that, using the same hormone preparation, a larger multiple of the dose is necessary to produce a significant increment of response with the former than with the latter method. On the other hand, the hypophysectomized rat is considerably more sensitive in its response to growth hormone than is the normal plateaued rat

The Tibia Test

The cessation of growth of the epiphysis following hypophysectomy in the dog and cat was first reported by Dott and Fraser (1923). It was later shown by Kibrick *et al.* (1941) that the epiphyseal cartilage response in young hypophysectomized rats to injections of increasing amounts of growth hormone over a 4-day period fell into a straight line dose-response curve when plotted on a semi-logarithmic scale. On the basis of these findings, the tibia test for the bioassay of pituitary growth hormone was proposed by Evans, Simpson *et al* (1943). The procedure, in brief, is as follows:—

Immature female rats maintained under standardized conditions are hypophysectomized at 26 to 28 days. The criteria for the completeness of hypophysectomy have already been mentioned. After a postoperative interval of 12 to 14 days, the animals receive daily intraperitoneal injections of growth hormone for four days. Twenty-four hours after the last injection, the animals are sacrificed with ether or chloroform, one or both tibiæ are dissected free from soft tissue, and split with a sharp razor in the midsagittal plane. The bone halves are stained immediately or fixed in 10 per cent neutral formalin. Prior to staining, the bone halves are washed in water

Increase in Weight of Normal Plateaued Rats

One of the first noted and most easily demonstrable effects of growth hormone is the acceleration of the growth of normal rats. The details of the procedure as used in this laboratory may be briefly outlined as follows:—

Adult female rats of the Long-Evans strain, 5 to 6 months old, weighing 220 to 280 g., in which growth stasis can be demonstrated by failure to gain more than 10 g. in 20 days, are satisfactory for the assay procédure. Male animals have not been as satisfactory as females. Diet and animal room conditions should be kept constant. The animals are weighed at 5-day intervals. Injections of hormone are made intraperitoneally daily for a specified period of time. Originally a 20-day test period was used, but a 15-day injection period has been found to be adequate. Growth hormone preparations of different potency tested by this method have produced log dose-response curves which are very nearly parallel, indicating that the response is reproducible. There is some variation in the responsiveness of the animals at different times of the year, so that it is essential to control assays with a standard hormone preparation, preferably the pure hormone, whenever possible.

Increase in Weight of Hypophysectomized Rats

Cessation of growth in the hypophysectomized rat and its resumption following implantation of whole pituitary glands was first demonstrated by Smith (1926, 1927, 1930). Among the first to utilize these phenomena in the assay of growth-promoting extracts of the anterior hypophysis were Van Dyke and Wallen-Lawrence (1930). The procedure for the hypophysectomized rat weight test as used in this laboratory may be described as follows:—

Immature female rats are hypophysectomized at 26 to 28 days of age, and, if evidence for completeness of operation has been obtained, are used for assay 10 to 12 days later. The criteria for completeness of hypophysectomy are: limitation of body weight gain to 7 g. in the pre-injection period,

established since the first report by Smith (1933) of this synergic action between the two hormones. On the other hand, the growth-promoting activity of growth hormone is known to be antagonized by the action of ACTH. The biological potency of growth hormone may therefore be expected to be modified by the presence of these hormones. Indeed, recent experiments in this laboratory indicate that the sensitivity of the tibia test can easily be altered by simultaneous injection with ACTH or thyroxine.

Although the specificity of the tibia test has been studied by Marx, Simpson *et al.* (1944), it has only recently been discovered that thyroxine alone causes a definite stimulation of the tibia width outside the range of non-specificity established by Marx *et al.* Furthermore, thyroxine enhances the potency of growth hormone (see Table I)

Table I

EFFECT OF THYROXINE AND ITS COMBINATION WITH GROWTH HORMONE (GH) ON THE WIDTH OF UNCALCIFIED CARTILAGE OF HYPOPHYSECTOMIZED FEMALE RATS

Experiment	*Total dose, μg*		*No of rats*	*Disc width micra*	*P values*	
	GH	*Thyroxine*				
I	0	0	36	166		
II	25	0	21	225		
III	50	0	14	242		
IV	0	8*	9	208 (19μg)‡	I-IV	<0 0001
V	0	26†	12	231 (46μg)‡	I-V	⩽0 001
VI	25	26†	15	253	II-VI	⩽0 001
VII	50	26†	16	273	III-VII	⩽0 001
VIII	50	8*	14	262	III-VIII	⩽0 001

*2 μg daily for 4 days, beginning 11th day after hypophysectomy
†2 μg daily for 13 days, beginning on the day of hypophysectomy
‡Values in parentheses indicate the GH equivalent as read from the GH standardization curve

There is some evidence that the thyroid of hypophysectomized rats may continue to manufacture and secrete a minute amount of thyroid hormone. If this is so it would be supposed that removal of the atrophied thyroid of hypophysectomized animals must lower their responsiveness to

for half-an-hour, immersed in acetone for at least one hour and then washed in water again for half-an-hour. They are then placed in freshly prepared 2 per cent silver nitrate for 1½–2 minutes, rinsed once in water, and while under water exposed to a strong light until the calcified portions appear dark brown. They are then immersed in 10 per cent sodium thiosulphate for 25 to 30 seconds, and washed in running water for half-an-hour. They are stored in 80 per cent ethanol in the dark. The width of the uncalcified epiphyseal cartilage is measured under the low power of the microscope, using a calibrated micrometer eyepiece. A minimum of 8 to 10 readings are taken and the results averaged.

An analysis of the method has been made by Greenspan *et al.* (1949). It was found that with groups of 8 to 10 animals, between dosage levels of 5 to 120 μg. of highly purified growth hormone per animal per four days, a straight line log dose-response curve was obtained. Over this range, an approximate doubling of the dose of hormone produced a significant increment in the response. The great advantage of the method is its high sensitivity. It was demonstrated that a total dose of 5 μg. of highly purified growth hormone was sufficient to produce a significant increase in the width of the cartilage plate in the hypophysectomized rat under the above conditions. This is about one-tenth to one-twentieth the quantity of the hormone necessary to produce a significant increment in body weight of the hypophysectomized rat in the 10-day test. A second advantage of the procedure is the short injection period, which is four days. It has been shown that any shorter injection time is unsatisfactory, but, on the other hand, increasing the injection period does not add to the sensitivity of the response.

Factors Influencing the Tibia Test*

That concurrent administration of thyroid hormone enhances the activity of growth hormone has been well-

*The data discussed in this section are taken from the unpublished work of Geschwind and Li, 1952.

nism is cancelled when thyroxine is present (see Table IV) Whether thyroxine counteracts the effect of ACTH directly or through its synergistic action of growth hormone remains to be investigated.

Table IV

INTERACTIONS OF GROWTH HORMONE (GH), ACTH AND THYROXINE IN THE TIBIA TEST

Experiment	*No of rats*	*Disc width micra*
Control	36	166
GH	21	225
GH+ACTH	16	193
GH+thyroxine	15	253
GH+ACTH+thyroxine	16	239

GH, 25 μg total dose, 2 μg thyroxine daily and 200 μg ACTH daily for 13 days, beginning on the day of hypophysectomy

Conclusion

From the foregoing account, it appears that the assay of the growth hormone activity of a crude preparation by any of the above-mentioned methods would offer great difficulties. The presence of minute impurities such as thyrotrophin (TSH), ACTH, etc., might result in an incorrect estimation of the actual potency of the hormone. It is therefore proposed that, for ideal results, hypophysectomized-adrenalectomized-thyroidectomized-gonadectomized animals should be employed for the bioassay of hypophyseal growth hormone.

REFERENCES

[illegible] 13, [illegible]

[illegible]

Endocrinology, 45, 455.

KIBRICK, E A., BECKS, H , MARX, W., and EVANS, H M. (1941) *Growth*, 5, 437

MARX, W , SIMPSON, M. E , and EVANS, H. M (1944). *Proc Soc. exp. Biol. Med.*, 55, 250

SMITH, P. E (1926). *Anat Rec* , 32, 221.

SMITH, P. E. (1927) *J. Amer. med Ass* , 88, 158.

growth hormone injections. This was found to be the case. It is evident from Table II that 50 μg. of growth hormone does not stimulate the tibia in hypophysectomized-thyroidectomized rats, whereas the same dose induces a good response in hypophysectomized animals. However, when thyroxine is injected in these double-operated animals, the responsiveness is restored.

Table II

THE EFFECT OF THYROIDECTOMY ON THE SENSITIVITY OF THE TIBIA TEST FOR GROWTH HORMONE (GH)

Experiment§	*Conditions*	*GH* μg	*Thyroxine* μg	*No of rats*	*Dose width micra*	*GH equiv* μg
I	H*	50	0	9	240	60
	H-T†	50	0	12	185	(0)
	H-T	50	8‡	6	223	31
II	H	400	0		295	(400)
	H-T	400	0	10	215	22
	H-T	400	8‡	5	253	105
III	H	50	0	5	252	105
	H-T	50	0	8	176	(0)

*H, hypophysectomized at 28 days of age
†T, thyroidectomized at 34 days of age or 6 days post-hypophysectomy.
‡2 μg thyroxine daily for 4 days simultaneously with the growth hormone injections
§Preparation L1672A was used in Experiments I and II, Prep L2265BP in Experiment III

The antagonistic action of ACTH to growth hormone as it affects the tibia test may be seen in Table III. This antago-

Table III

EFFECT OF ACTH PLUS GH ON THE WIDTH OF UNCALCIFIED CARTILAGE OF TIBIA OF HYPOPHYSECTOMIZED FEMALE RATS

Preparation	*Total dose** mg	*No of rats*	*Average width of uncalcified cartilage* μ
GH	0·05	9	224·4±8·0†
ACTH	4·0	10	115·6±5·5
GH+ACTH	0·05+4·0	10	142·8±6·2
Uninjected Controls	—	10	163·2±4·6

*Injected over a 4-day period into female rats, starting 10–14 days after hypophysectomy at 26–28 days of age The ACTH was given in three daily doses
†Mean ± standard error

to see if the *in vitro* effects of growth hormone could be employed as an assay technique.

FOLLEY We have found a good dose-response relationship for the stimulating effect of insulin on fatty acid synthesis in mammary gland slices *in vitro*, and I would imagine that with a constant amount of insulin you would probably get a dose-response relationship for the antagonistic effect of growth hormone on the insulin effect.

LI That would certainly be an ideal assay method.

FOLLEY: The only thing that's rather troublesome is the extraction of the fatty acids, which takes a couple of days; and you need counting equipment, of course.

ROBSON: Dr Li, have you any evidence whether ACTH inhibits the effect of thyroxine on the thyroid? It seemed to me that the ACTH presumably inhibited the effect of growth hormone on the thyroid, and it reminded me of those findings of Reiss, of ACTH inhibiting the effects of gonadotrophins I wonder if there is any evidence that there is an effect of ACTH on the target organ for thyroxine.

SEGALOFF: May I ask a similar question? Here we are thinking of using this

growth ho

happens if

the growth

if we have a preparation beyond a certain degree of purity, can we ignore the contamination with ACTH and thyrotrophin?

LI: Since only very small amounts of thyroxine are needed to produce a very marked effect, it would be expected that a TSH contaminant in a growth hormone preparation would be a factor which would interfere seriously with an accurate estimation of the amount of GH activity It might also be expected that if the contaminant were ACTH, a very small amount might actually act synergistically with growth hormone, whereas a larger amount does inhibit the growth hormone activity The quantitative relationship of these contaminants as they influence

extract, given with the growth hormone, can remarkably diminish its effect.

SMITH, P. E. (1930). *Amer. J. Anat.*, 45, 205
SMITH, P. E (1933). *Proc. Soc. exp. Biol. Med.*, 30, 1252.
VAN DYKE, H. B., and WALLEN-LAWRENCE, Z. (1930). *J. Pharmacol*, 40, 413.

DISCUSSION

REID I wonder if Dr. Li has found variations in sensitivity from one run to another. The use of a unit based on an absolute response I think is warranted only if the sensitivity is constant. And I do find that with intact rats their sensitivity varies quite considerably from test to test

in 1942. So that the amount of hormone needed to get an assay of a given accuracy is not so great as one might anticipate

I'm very interested in this effect of thyroxine and thyroidectomy in the tibia test Is it correct that the body weight increases are not influenced by thyroidectomy?

LI: No The body weight is also changed by thyroidectomy. I think the basic fact is that growth hormone is growth-promoting even in thyroidectomized animals, but they require a higher dose. The tibia test, too, is less sensitive in thyroidectomized animals.

REID: You haven't tried adrenalectomized animals?

LI· This is always the case—the growth hormone promotes body

With purified growth-hormone preparations, 5 mg./day is a suitable dose in a typical cat. If no response is obtained after six days, the dose is tripled. When the response is obtained and injections are stopped, the urine becomes sugar-free in one to four days, and the animal is then rested for at least six days before the next test is commenced.

Relationship between Dose and Time to Induce the Response

A series of tests with a sterile crude pituitary extract in Cat 117 is shown in Fig. 1. Despite a tendency for sensitivity to diminish, discrimination between different doses was obtained, at least with those which gave the response only after five or more days of treatment. Since similar discrimination

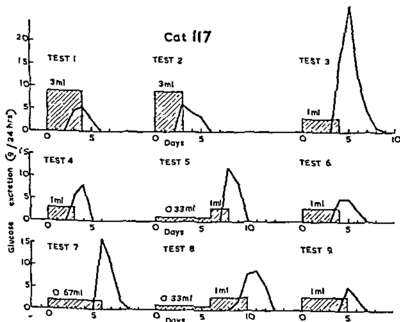


FIG. 1. Tests with a crude pituitary extract in Cat 117 Period of treatment denoted by hatched rectangle, the height of which is proportional to the daily dose (also indicated numerically).

ASSAY OF GROWTH HORMONE FOR DIABETOGENIC ACTIVITY

E. REID

YOUNG (1945) found that the intact cat resembled the intact dog in its suitability for testing diabetogenic pituitary preparations, although the cat was more prone to lose appetite during testing. Despite this disadvantage, the cat has proved to be suitable (Reid, 1951*a*) for quantitatively determining the diabetogenic activity of beef anterior pituitary extracts, or of growth hormone itself, which appears, from our present evidence, to be the main active constituent of such extracts. In comparison with the intact cat, the partially depancreatized or alloxan-diabetic rat is rather insensitive as a test animal for this purpose, and indeed is qualitatively different since it responds more readily to ACTH than to growth hormone (Bennett and Li, 1947; cf. Reid, 1951*a*, 1951*b*).

The assay method is based on the finding that there is, with a given test preparation in a given cat, an inverse relationship between the dose and the period of treatment required to induce mild temporary diabetes. This relationship can be demonstrated quite consistently, with the practical limitation that most cats become refractory (insensitive) sooner or later during a prolonged series of tests.

Experimental Conditions

The tests are performed in healthy adult cats, preferably male (or male castrate), which are maintained on a meat diet (Young, 1945; Reid, 1951*a*). Food consumption and urine output are measured daily. Injections of the test preparation are given subcutaneously once daily, at a dose which is considered likely to induce a 24-hour glucose excretion exceeding 5 g. ("5 g. response") after six days of treatment.

to a value of unity for the ratio (initial dose/6-day dose). Only in the case of some responses obtained in four days or less were there marked divergences from this linear relationship. Data obtained from more recent tests have now been similarly plotted (Fig. 2), the straight line being positioned exactly as before (Reid, 1951*a*). It will be noted that these

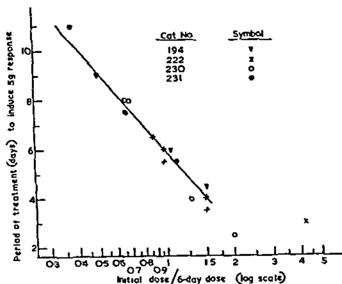


FIG 2 Relationship between dose of growth-hormone preparations and period of treatment required to induce diabetic response in intact cats

more recent data, especially those for responses obtained in 5–8 days, are satisfactorily represented by the line based on the earlier data. It appears, then, that the relationship found originally in Cat 117 applies to any cat under our test conditions, provided of course that a refractory state does not develop during a series of tests

Routine Assay Procedure

In routine assays the six-day dose of each test preparation is obtained directly if the dose was such that the response

was found with other diabetogenic preparations in this ca[t] an assessment of the relative potency of two preparation[s] could validly be based on determinations of the respectiv[e] doses which were effective in a given time, say six days. I[f] moreover, the period of treatment required to obtain the 5 g response bore a constant relationship to the dose, then fo[r] any test preparation the daily dose required to induce the response in six days ("6-day dose") could readily be calculated from that employed in a test in which the response was not obtained in exactly six days.

The following procedure was adopted (cf. Fig. 2) to ascertain whether the relationship could validly be regarded as constant, not only for different test preparations, but also for different cats varying in absolute sensitivity. In each of a series of tests (cf. Fig. 1) the "6-day dose" was found empirically, or was estimated roughly from the doses administered (if none of the responses occurred in exactly six days) by interpolation or extrapolation over a small range. The dose employed initially in each test was then expressed as a fraction of this six-day dose, and the logarithm of this ratio plotted against the number of days required to induce the response. It is evident that any small error in estimating the denominator—the 6-day dose—in a series of tests would affect only the position, and not the slope, of a line joining the points plotted for that series. Since anorexia during a test delays the appearance of the response, data from tests in which there was severe anorexia were discarded. If, however, there was mild anorexia on, say, one day in a test in which the response occurred in x days, it seemed reasonable to plot the point at $(x-\frac{1}{2})$ days instead of at x days. A similar correction was applied if the 5 *g.* response was "non-typical" in the sense of being either unusually large (exceeding 15 *g.*) or preceded by a glucose excretion just short of 5 *g.*

The points thus obtained, for numerous test series in several cats, were found (Reid, 1951*a*) to fit a straight line which was, of course, positioned so that six days corresponded

The latter criterion is fulfilled if the actual response in each test is obtained in about 6 days (5–8 days), and if the food intake is almost constant throughout. Success in keeping food intake constant depends not only on restriction of the daily food ration, but also on stimulation of appetite, when necessary, by suitable persuasive measures! Fuller consideration has been given elsewhere (Reid, 1951*a*) to criteria for roughly assessing the relative reliability of different potency values. To gather enough data for rigorous statistical treatment would probably be a formidable task.

Table II gives some potency values which were determined in duplicate, excluding any assays in which the conditions were considered poor. There is fair agreement between duplicate values obtained for the relative potency of any pair of test preparations, these including crude anterior pituitary extract (beef), purified growth hormone, and a growth hormone preparation subjected to an inactivating treatment.

Table II

DUPLICATE DETERMINATIONS OF RELATIVE POTENCY

Batch nos of test preparations		*Relative potency, A/B*	
A	*B*	*1st assay*	*2nd assay*
279A	276	0 82	0 96
284	276	2 1	1 4
292	17 GH enz	17	17
292	20 GH	14	11 5
293	(21, 22) GH	7 0	5 0
242	24 GH	3 0	2 8

In conclusion it may be emphasized that the intact cat is one of the few animals suitable for testing growth hormone preparations for diabetogenic activity, and that assays in the cat are slow and require close attention to experimental conditions; but otherwise the assay is simple Its specificity and sensitivity remain to be considered. The response in the cat is specific for growth hormone, with the reservation that the effect may be due to a factor closely associated with

was obtained in six days; otherwise it is calculated from that actually used, by formulæ (Table I) based on the above relationship. A small correction may be applied if there is mild anorexia, or if the 5 g. response is "non-typical" in the sense stated above. If in such a test the response were obtained in six days with a dose of 10 mg./day, the six-day dose could reasonably be assessed as 9 mg. instead of 10 mg.

Table I

ASSESSMENT OF "6-DAY DOSE" FROM A SINGLE TEST UNCOMPLICATED BY ANOREXIA OR "NON-TYPICAL" RESPONSE

Daily dose	*Days of treatment to elicit "5 g response"*	*6-day dose*
x	1, 2 or 3	<0 65x
x	4	0 65x
x	5	0 8 x
x	6	x
x (6 days), 3x (1 day)	7	1 25x
x (6 days), 3x (2 days)	8	1·55x
x (6 days), 3x (3 days)	9	1 95x
x (6 days), 3x (4 days)	10	2 4 x
x (6 days), 3x (5 days)	11	3·0 x

The six-day dose determined for any test preparation refers only to a particular test in a given cat, and is in no sense an absolute "animal unit". It is used merely as a basis for comparing the potency of the preparation with that of a reference preparation. Such a comparison may be made by performing "cross-over" tests in two cats (Reid, 1951*a*), or consecutive tests in a single cat:—

Test preparation	*6-day dose*	*Relative potency, A/B*
B (initial test)	b_1	
A	a	$\frac{b_1 + b_2}{2a}$
B (final test)	b_2	

For a satisfactory assay by the above procedure, it is clearly desirable that b_2 should not be substantially different from b_1 (i.e. little change in the sensitivity of the cat), and that a, b_1 and b_2 should be known with reasonable certainty.

Astwood The effect on recently induced, but existing diabetes, determining the rate at which the diabetes goes away.

Reid: Unfortunately that works the opposite way. A very high dose is required, at least in some cats, to exacerbate or to maintain glycosuria when it is already there.

Astwood With a threshold dose, will the diabetes appear and then pass off?

Reid: If one obtained a small response on the sixth day, shall we say, and continued to inject the same dose, the response probably would tail off. That is why I think it is desirable to increase the dose after six days

was no marked change in blood sugar at 1½ and 3 hours after injection of growth hormone. That's all the information I have.

Fraser. Do they diminish the hypoglycæmia of insulin in proportion to this diabetogenic activity?

Reid: I have not studied that, but I think that conclusion is implicit in the work in Wilhelmi's laboratory with rats, and in Young's earlier work with dogs.

Gaddum: Could you make the cats more sensitive by giving them a little phlorrhizin?

Reid: One should be able to One can pick up a slight rise in blood sugar quicker than a glycosuria. I have a definite impression, however, that a response based on a certain blood sugar level is less closely related to the dose than the glycosuric response

Gaddum: If you gave enough phlorrhizin to cause a small glycosuria then you might expect that a small dose of growth hormone would increase it.

Reid: Yes. I haven't tried that.

Vogt: Is there some reason why in cats the male seems to be more favourable in these tests, and why in the growth hormone tests Dr. Li has been talking about, it's always female rats?

Reid In the cat tests I prefer to work with males because it is not known, and has not been systematically studied, whether an animal's coming on heat does affect the response Females, when we have had to use them, have worked quite well

Li In our case we have always used the hypophysectomized animal whether it is male or female Male rats are unsuitable for studies with intact animals because they continue growing, no matter how old they are.

Astwood. Could you compare the relative merits of the dog and the cat in this assay?

or about 2.

growth hormone. The response also appears to be unaffected by the presence of other pituitary hormones, with the important exception of ACTH, which can enhance the diabetogenic action of growth hormone preparations although it is not diabetogenic itself (Reid, 1951*c*). This enhancing action of ACTH might be exploited in testing ACTH preparations for contamination with growth hormone, which might escape detection in a conventional growth-hormone test because of inhibition by ACTH.

In an exceptionally sensitive cat a test may be performed with a total of 15 mg. of growth hormone. Possibly this rather large requirement could be reduced somewhat by giving divided injections, or by delaying absorption in some way. A third possibility would be to "prime" the cat with ACTH, which may under some circumstances produce a two-fold increase in sensitivity which may persist for over two months. It is doubtful, however, if the hormone requirement could be reduced much below 5 mg. It thus seems unlikely that the assay procedure could be applied to body fluids, as the clinical investigator would desire.

[illegible] during the tenure of a Carnegie [illegible] of the experimental animals.

REFERENCES

Bennett, L. L., and Li, C. H. (1947). *Amer. J. Physiol.*, 150, 400.
Reid, E. (1951*a*). *J. Endocrinol.*, 7, 120.
Reid, E. (1951*b*). *J. Endocrinol.*, 7, xxxvi.
Reid, E. (1951*c*). *Nature, Lond.*, 168, 878.
Young, F. G. (1945). *Biochem. J.*, 39, 515.

DISCUSSION

Astwood. Is it possible to get some rough idea of potency with smaller [illegible] and then in that same [illegible] effect on existing diabetes?

THE ACTION OF PITUITARY PREPARATIONS ON THE ADRENAL CORTEX

M. P. STACK-DUNNE

THE work here described is part of a programme of research into the properties of ACTH carried out at Cambridge under the direction of Professor F. G. Young. Dr D. B. Cater has carried out the histological work, and Mr H. B. F. Dixon has collaborated very fully in the other investigations.

I wish to discuss our experiences in the biological identification and estimation of ACTH, and the interpretation of the data obtained. The methods we have used have depended upon direct examination of the adrenal, following acute treatment of the animal with the test preparations As yet, no attempt has been made to study the adrenal secretion directly, but we hope to do so in future work All rats used in the study of adrenal function have been hypophysectomized adult male rats of a Wistar strain.

In recent years, the test most commonly employed for ACTH has been the adrenal ascorbic acid depletion test (Sayers, Sayers and Woodbury, 1948), and this with good reason, in view of its speed, precision and specificity. This test has formed our chief criterion for ACTH and the basis of comparison for other methods of testing. As this test is well known I need mention only points of special interest. We find with our strain of rat that the body weight must be above 200 g to avoid undue variation. It is also essential that the rats have abundant drinking water after operation; lack of water strikingly lowers the sensitivity. Fig 1 shows a log-dose response curve obtained from tests performed over a period of three weeks. There is a marked scatter of the points in the region of the curve corresponding to a dose of 0·125 µg. of the standard per 100 g. body weight of rat.

BUTTLE: Did you find that thyroxine made no difference?

REID: Thyrotrophin in two experiments did not have any effect on the activity of growth hormone.

BUTTLE: You didn't try thyroxine?

REID: No.

INGLE: Dr. Frank Engel told me recently that the normal rat, which does not develop glycosuria when given growth hormone by itself, does develop glycosuria when treated with thyroxine and growth hormone in combination. This has not been published. He has also recently published data showing that if the rat is given a subdiabetogenic amount of corticotrophin, the addition of growth hormone will cause glycosuria. Dr. Li and I have similar findings in experiments that are under way at the present time.

LI: Have you confirmed the observation of Campbell in Toronto on permanent diabetes, produced by one injection of growth hormone?

REID: I had the impression that it was several injections, but I may be wrong. We try to avoid permanent diabetes, but rarely we do induce it by accident, by pushing the cat "over the brink" in some way.

BUTTLE: How long would it take to produce that?

REID: In the most rapid example we have had, diabetes developed after, I think, the third injection in a series of tests; instead of the glycosuria tailing off, it became permanent.

COX: What sort of interval do you find it desirable to leave between these tests to prevent such after-effects?

REID: A period of at least six days of sugar-free urine. This permanent diabetes has been an extremely rare phenomenon, so that interval is generally adequate.

COX: Have you tried giving the same dose at successive intervals to the same animal, to see if there were any signs of the response changing progressively with time?

REID: I have not done that systematically, but I have some data which suggest that 6 days is as good as 16 or 26.

MACLAGAN: This is quite a minor point, but how do you "persuade" your cats to eat?

REID: The simplest method is to avoid having stale diet exposed to them, by feeding them twice a day. But if a cat is beginning to look "broody" about its food, one can quite legitimately give the animal not only the usual minced diet (mainly cooked liver and raw beef) but some bits of the raw meat alone, which most cats find more palatable. If one is desperate, and wants to save an important assay, one can try milk, which after all contains cow protein. These measures are usually successful if they're assiduously pursued.

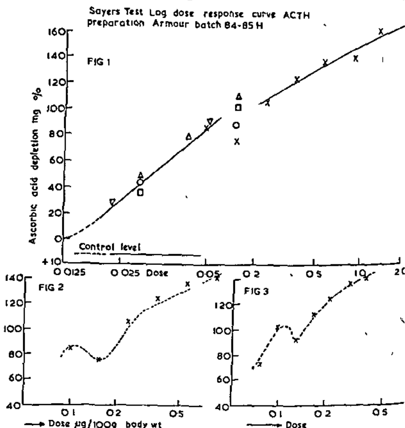
only 5 to 10 per cent would cause an apparent increase of the ascorbic acid level sufficient to account for the observations. The dose response relation has been studied most extensively using Armour ACTH batch 84–85 H (3·45 times the potency of La-1-A), which is a partially hydrolysed preparation, but the effect has also been observed when using a crude ACTH prepared by the method of Astwood and colleagues (Payne, Raben and Astwood, 1950).

A second method of assay which we are using is an adrenal weight repair method. In this the test animals are injected intraperitoneally three times a day for three days on the 18th to 20th day after hypophysectomy and killed on the 21st day. The adrenals, testes, and thymus are weighed. As a rule the pituitary extracts were treated before injection with tannic acid in the presence of an inert protein. This increases the sensitivity of the test about five-fold, probably by slowing the rate of absorption. It also reduces the possibility of anomalous results which might arise from differences in absorption rate. The technique is as follows: for each rat 0·45 ml. of the test solution and 0·025 ml. of 1 per cent serum albumin are mixed in the syringe, followed by 0·025 ml. of 1 per cent tannic acid, the mixture is gently shaken until precipitation is complete, and then injected. The sample solutions are prepared in physiological saline containing 0·25 per cent acetic acid and 1 per cent thiodiglycol and are kept at 4°C. during the test period.

Recently, we have tried the method for delaying the absorption of ACTH preparations suggested by Dr. Parkes (Bruce and Parkes, 1952). The dry sample is suspended in 5 per cent beeswax in arachis oil. This method was found to give a potentiation of at least twenty-fold, being much more effective than the tannic acid technique.

We find the adrenal weight test almost as reliable as the Sayers test. Groups of four or five rats are adequate, but there appears to be a tendency for variations to increase when the final weight of the adrenal is greater than that of the unoperated animals.

When the results obtained on any one day are examined there is frequently seen a decrease in response for an increase in dose in this region. Figs. 2 and 3 show this effect clearly.



FIGS. 1–3. Log dose/response curve for ACTH Armour batch 84–85H in the ascorbic acid depletion test.

Five to six rats have been used per dose level. The depression in the curve appears to be most marked when the initial ascorbic acid content of the adrenals is above average. A possible explanation of the phenomenon is that the acutely stimulated adrenal suffers a slight fall in weight. This needs further investigation, but a fall in the adrenal weight of

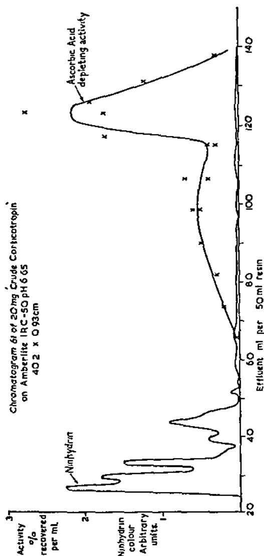


FIG 4. Chromatogram of "crude corticotropin" on Amberlite IRC-50

The histological techniques used were as follows: Frozen sections from adrenals fixed in Baker's formol calcium were stained for lipids with Sudan black or Scharlach R. Unstained sections were examined with the polarizing microscope for birefringent material. Schultz's method for cholesterol was also applied. For mitotic activity, adrenals were fixed in Suza, embedded in wax, serial sectioned and stained with hæmatoxylin and eosin. Representative mid-sections were projected by a camera lucida on to squared paper and the area of the cortex measured. A sufficient number of adjacent sections were examined for mitotic figures to make up an area of 12 mm ^{2}, and the positions charted on the camera lucida drawings.

These methods have been applied to rats' adrenals after treatment with a variety of pituitary preparations, and some of the results have already been published (Stack-Dunne and Young, 1951; Dixon, Stack-Dunne, Young and Cater, 1951; Cater and Stack-Dunne, 1952). I would like to summarize the results of these investigations, and as far as possible to correlate them We must apologize for so much of the data being incomplete.

Using the protein fractionation techniques developed by Moore, Stein and colleagues, a number of distinct protein and activity fractions have been separated from crude corticotrophin by chromatography on columns of the ion exchange resin Amberlite IRC-50, under the conditions we have previously described (Dixon, Moore, Stack-Dunne and Young, 1951). More recent work using the same system has given rather better resolution of the components and this is shown in Fig. 4. The main proteins are fast moving, and have separated into a number of components. Examination of these by the adrenal weight test shows all of them to be active, and collectively to contain about 50 per cent of the adrenal weight activity put on to the column. The ascorbic acid depleting activity is resolved into two peaks, which on rechromatography retain their characteristic positions. These contain, as did the originally unresolved peak, slight, but definite, adrenal weight activity. As yet we have no indication that the two ascorbic acid depleting peaks, or the smaller portion which is eluted from the column only with alkali, can be distinguished from one another by physiological

hormone, which is extracted under similar conditions, contains somewhat more A.A. activity, but is still much more effective in the adrenal weight test. In both these preparations the ratios of A.W. to A.A. activity are so high in terms

Table I

A comparison of the Adrenocorticotrophic activities of Armour ACTH Batches 84-85H and 84-85U in the Adrenal Weight Repair and Ascorbic Acid depletion tests.

1 Assay in Ascorbic Acid depletion test
84-85H = 3·45 La-1-A
84-85U = 2 5 La-1-A = 0·72 84-85H.

2. Assay in Adrenal weight repair test.

SAMPLE	DOSE	No RATS	MEAN PAIRED ADRENAL (mgms) ± S e	INJECTION PROCEDURE.
84 - 85 H	15 mg	4	14 2 ± 0 5	Injected in Physiological Saline 0 25% Acetic acid
84 - 85 H	45 mg	4	16 9 ± 1 50	
84 - 85 U	5 mg	4	17 5 ± 0·40	
84 85 U	15 mg	3	21 6 ± 2 50	
84 - 85 H	1 0 mg	5	15 3 ± 1 0	As above but with addition of 0·2 % Serum Albumen and 0 2 % Tannic acid
84 - 85 U	1 2 mg	5	17 2 ± 0 8	
84 - 85 H	0 5 mg	5	14 5 ± 1 4	Injected as suspension in 5 % Beeswax-Arachis Oil 2 injections per day each 0 2 ml intraperitoneally
84 - 85 H	1 5 mg	5	18 9 ± 1 4	
84 - 85 H	10 0 mg	5	27 5 ± 1 2	
84 - 85 U	0 05 mg	5	11 9 ± 1 2	
84 - 85 U	0 15 mg	5	12 6 ± 0 8	
84 - 85 U	1 0 mg	3	22 3 ± 1 3	

of the standard, in this case 84–85 U, that it seems unlikely that differences in the rate of absorption can be the correct explanation.

Gonadotrophins

Many workers have considered that gonadotrophins have a direct effect upon the adrenal cortex (Miller and Riddle,

methods, as judged from their behaviour in the Sayers test, or in preliminary histological studies.

An examination has been made of the mitotic activity in the adrenals of the rats used for the adrenal weight test on the fractions from the columns. This has shown that the fast moving proteins are also associated with the main mitotic stimulating activity. The separation of most of the adrenal weight and mitotic stimulating activity on the one hand from the ascorbic acid depleting activity on the other is clear cut.

Differences between Unhydrolysed and Hydrolysed ACTH Preparations

We have observed differences in the ratios of the adrenal weight (A.W.) activity to the ascorbic acid depleting (A.A.) activity of the Armour preparations 84–85 H and 84–85 U. 84–85 H has been prepared from 84–85 U by a process involving hydrolysis. The experimental data on these two preparations are shown in Table I. A more rapid absorption of the hydrolysed 84–85 H seemed a possible explanation of the differences, but all attempts to delay absorption have potentiated both preparations more or less uniformly. In view of the different fractions obtained by ion exchange columns it seems more likely that most of the adrenal weight activity of 84–85 H has actually been destroyed, perhaps by hydrolysis. It should, however, be noted that the A.W. activity is not destroyed by mild acid treatment (0·1 N HCl at 37°C., or 0·025 N HCl at 100°C.).

Extracts Exposed to the Action of Pituitary Enzymes during Preparation

Pituitary tissue suspended in neutral or alkaline aqueous media rapidly loses its ascorbic acid depleting activity. Thus crude alkaline extracts of ox anterior pituitary tissue are very low in ascorbic acid depleting activity; our preparations contain less than a ten-thousandth part of the activity originally present in the gland. However, they have a considerable adrenal weight effect. It was found that growth

The Evidence for More than One Adrenocorticotrophic Factor

If one examines by histological means the adrenal cortex of a normal unstressed rat, one finds much lipid, and considerable mitotic activity, but little evidence of secretory activity. Other evidence shows that the normal adrenal can be releasing only small quantities of cortical steroids. If the pituitary is removed, the following picture results: there is a marked increase in the width of the transitional zone, to give the well known "sudanophobe zone"; a considerable quantity of lipid remains in the other parts of the cortex (at least in the first weeks), and of course there is no evidence of synthesis or secretion. Virtually all mitotic activity ceases. The question that faces the experimenter is how to return such an adrenal to the normal picture, and the concept of the hormone will rest on the results of this attempt There have to be considered, in addition, the phenomena which can be observed in the stressed intact animal· marked loss of lipid in the initial stages (this is progressive if the stressful conditions are maintained); and evidence of vigorous synthetic activity. It must be possible to obtain this picture also. If only one hormone is postulated, then by suitably modifying the level of dosage, it must be possible to return at least approximately to either condition from that found in the hypophysectomized rat.

In our experience, administration of growth hormone preparations to hypophysectomized rats will return them to a state approaching normal, as judged by mitotic activity and lipid distribution On the other hand any quantity of the preparation 84–85 H sufficient to promote appreciable regeneration of cells will also cause the changes in lipids that are associated only with the normal animal when under stress.

This forces us to suggest as the most reasonable explanation of the experimental results that the factor responsible for maintaining the adrenal in the normal condition is best identified with the activity found in the growth hormone

1939; Golla and Reiss, 1942; Greep and Jones, 1950). Opsahl and Long (1951) have shown that a preparation can be obtained from human placenta that is effective in stimulating the adrenal, as judged by the inhibition of the hyaluronidase spreading effect. It would appear from their data that this preparation is deficient in the Sayers test. We have examined human chorionic gonadotrophin, and placental preparations made by the method of Opsahl and Long, and find that they are effective in the adrenal weight test but have little activity in the Sayers test. Histological examination of this material is not yet complete and I would not like to comment further on the data.

Adrenal Mitotic Activity of Growth Hormone and Armour Preparation 84-85 H

We have paid special attention to a comparison of the mitotic stimulating activity of purified growth hormone and 84–85 H, two preparations which were conveniently available and showed extreme differences in the adrenal weight and Sayers tests. The number of mitotic figures in 12 mm.^{2} of adrenal cortex numbered 1 to 6 in the hypophysectomized rat, 7 to 12 in rats treated with 0·5 mg. of 84–85 H, 25 to 46 in normal rats, and 50 to 94 in rats treated with 20 mg. of growth hormone. More recent unpublished experiments have shown, in addition, that simultaneous injection of 84–85 H and growth hormone at these dose levels gives mitotic counts of essentially the same order as the growth hormone acting alone. The stimulation is most marked in the zona glomerulosa and outer fasciculata but is also found deeper in the cortex. No characteristic differences in distribution of the mitoses have been noted with any of our preparations. When the preparation of growth hormone and 84–85 H were given simultaneously, there was some evidence of synergism in adrenal weight increase but none in the mitotic stimulating activity itself.

test. However, since in the normal animal it is probably the A.W. factor which is effective in maintaining the adrenal weight there is some excuse for the term, and to avoid confusion we would prefer to retain it.

We have not sufficient data at present to state whether the A.W. factor can be identified with growth hormone itself, especially as the properties of growth hormone from pig pituitaries may not be identical with that from beef glands. Our growth hormone preparations have always caused a marked increase in testis weight and, in view of the results with chorionic gonadotrophin, the implication of gonadotrophins in the effect also must be considered.

REFERENCES

[illegible] Lancet, 262 (No. 6698), 71.
C [illegible] (1952) Communication to

[illegible], MOORE, S, STACK-DUNNE, M P., and YOUNG, F G. (1951). *Nature, Lond.*, 168, 1044.
[illegible] ING, F G., and CATER, D B.

C [illegible] *Endocrinol.*, 3, 5.
G [illegible] *posium on Steroid Hormones*,

MILLER, R. A., and RIDDLE, O (1939) *Proc. Soc. exp Biol. Med*, 41, 518
OPSAHL, JEANETTE, C., and LONG, C. N. H. (1951) *Yale J. Biol Med*, 24, 199
PAYNE, R. W., RABEN, M S., and ASTWOOD, E B. (1950). *J. biol. Chem.*, 187, 719
SAYERS, M. A., SAYERS, G., and WOODBURY, L A (1948) *Endocrinology*, 42, 379
STACK-DUNNE, M P., and YOUNG, F. G. (1951) *J. Endocrinol*, 7, lxvi.

DISCUSSION

VOGT [illegible]
a
[illegible]
a
a
t

preparations; while a second factor, secreted in appreciable quantities only in stress, is best identified with the activity found in the preparation 84–85 H and, by inference, with the ascorbic acid depleting activity. Our other observations can be explained in terms of these concepts. It is reasonable to identify with the activity found with the growth hormone: (1) the activity associated with the faster running fractions obtained when unhydrolysed pituitary preparations are fractionated on the ion exchange columns; and (2) the activity that is preferentially lost or destroyed in the preparation of 84–85 H from 84–85 U.

The ascorbic acid depleting activity, which is virtually absent from the growth hormone, is common to the more basic fractions from the ion-exchange column and to the 84–85 U and the 84–85 H. So far, it has not been possible to obtain from preparations originally highly active in the Sayers test any fraction which has retained its growth hormone-like activity and at the same time has quite as low an ascorbic acid depleting activity as our growth hormone preparations, although the fractions from the columns have gone far towards this end.

To consider terminology, we would still like to use the terms A.W. activity for the activity found typically *in the* growth hormone preparations, and A.A. activity for that measured in the Sayers test. *Hence A.A. activity corresponds* to what has usually been considered ACTH. The *A.W. activity is to be associated principally with the mitotic*-stimulating activity but, as far as we can tell at present, with *some lipid changes also, especially return of* lipid to the sudanophobe zone, although this lipid may not be fully *typical. The A.A. activity is to be associated* with the depletion of ascorbic acid, and probably with the stimulation of *steroid* secretion. The term A.W. activity is not entirely suitable, because weight increase can be due to hypertrophy of cells or engorgement of blood vessels in addition to hyperplasia, and hence the A.A. factor will be expected always to show some activity in the so-called adrenal-weight repair

Cater. We do get repair of the sudanophobe zone with growth hormone. We have always been very cautious about saying that this effect upon the adrenal is an intrinsic property of the growth hormone. It may of course be something in the growth hormone, and it rather looks as though the results of ultrafiltration would be very strong evidence in favour of that view. Of course our growth hormone pre-

tissues of the rat.

P. Morris We have given two patients ultrafiltrable ACTH which was very potent in adrenal growth factor as well as the ascorbic acid factor. In terms of the Sayers test I should say it was roughly equivalent to 50 mg. a day of LA-1-A. We have increased the ketosteroids to something of the order of 200 per cent quite quickly. Whether that's associated with the adrenal weight factor or the ascorbic acid factor, there is no evidence on which to decide.

Stack-Dunne We have wondered whether the preparations which have proved effective in treating patients could be improved with the adrenal weight factor, especially when you're treating patients for a long time

be

an

rich in adrenal weight factor—I say "impression" because there weren't very many patients—the effects were much more prolonged than we would have expected from an equal A.A dose.

Segaloff: Have you measured the ascorbic acid in the adrenals after a long time, particularly after using the preparations with delayed absorption?

Stack-Dunne· The ascorbic acid level of the hypophysectomized rats falls to about half that of the normal, and you can lower that further with a dose of ACTH.

Fingl. We continue to have difficulties with assays of the various fractions

assay

the sa

consid

tration employed, and number of injections

Stack-Dunne It can't be eliminated as a possibility, but we don't think it's likely. We're trying to get around it all the time.

Fingl: Perhaps the adrenal ascorbic acid depletion test could be conducted over a 3- to 5-day period.

Stack-Dunne· It might be worth doing.

acid activity there, it is difficult to assess the importance of weight

tomized when they're about 120–150 grams. We take them all at the same age, so there's some spread in the weight of the animals.

LORAINE: Is there any change in sensitivity when rats weighing over

essentially a property of growth hormone?

STACK-DUNNE That we don't know Some recent work on growth hormone preparations suggests it may not be.

C. J. O. R. MORRIS We rather feel that it isn't We can ultrafilter the adrenal weight factor, and we know that it's impossible to ultrafilter growth hormone activity under those conditions.

STACK-DUNNE: That's a little difficult to interpret, isn't it, because you concentrate the growth hormone activity after it has come through a different extraction procedure?

ATTEMPTS TO ASSAY ACTH WITHOUT HYPOPHYSECTOMY

G. A. H. BUTTLE and J. R HODGES

SAYERS, Sayers and Woodbury (1948) showed, in hypophysectomized rats, that the adrenal ascorbic acid depletion produced by the intravenous injection of ACTH was proportional to the dose of ACTH administered. They developed this finding into a sensitive method for the bioassay of ACTH. A disadvantage associated with the use of the adrenal ascorbic acid depletion technique for the biological standardization of the adrenocorticotrophic hormone is the fact that only hypophysectomized animals can be used. In normal animals any form of stress causes the secretion of endogenous ACTH. If an attempt were made to carry out the Sayers' method in normal animals, no results would be obtained, since the stress of the surgical removal of the control adrenal gland would result in maximal adrenal ascorbic acid depletion in the other. The administration of ACTH could produce no further effect.

Sayers and Sayers (1947) and Long (1947) showed, in rats, that the fall in adrenal ascorbic acid concentration which follows the application of stress could be prevented by pre-treatment of the animals with adrenocortical hormones. These findings suggested that it might be possible to modify the Sayers' method by replacing hypophysectomized animals with rats in which the mobilization of endogenous ACTH had been prevented by pre-treatment with adrenocortical hormones. Our attempts to effect "pituitary block" with deoxycorticosterone acetate are described here.

Rats were given intraperitoneal injections of deoxycorticosterone acetate in arachis oil. At various time intervals after receiving the DCA injections the animals were anæsthetized

[illegible]

extended these experiments.

LI: This has been published in a review paper in *Acta Endocrinologica*, 1951, 8, 393 (Reinhardt, W. O., Geschwind, I. I., and Li, C. H.).

STACK-DUNNE: Do you think it is an absorption phenomenon entirely?

LI: No.

BUSH: It would look from Dr. Li's results as though preparations containing a high proportion of adrenal weight activity did in fact cause a considerable increase in cortical secretion, since the thymus weight effect should be the most certain indication of increased adrenocortical secretion.

STACK-DUNNE: But you do run into the trouble here that the adrenal weight estimation is a rather arbitrary one. You may get cell hyperplasia or vascular engorgement. The adrenal weight test does not necessarily estimate only what we like for the moment to call "A.W. factor".

It was found that control injections of normal saline produced no adrenal ascorbic acid depletion in DCA-treated rats. Intravenous ACTH caused a fall in the adrenal ascorbic acid concentration. Over a range of doses of ACTH equivalent to 0·08 μg. to 1·25 μg. International Standard, there existed a linear relationship between the logarithm of the dose and the adrenal ascorbic acid depletion. Doses of ACTH greater than 1·25 μg. produced no larger responses.

The mean value obtained for the index of precision of the method ($\lambda = 0 \cdot 463$) was similar to that obtained in our own laboratory using the Sayers' method for the bioassay of ACTH, and that reported by Greenspan, Li, Simpson and Evans (1950) using hypophysectomized animals. We have been unable to obtain the precision which many other workers, using the Sayers' method, have reported. However, all the animals used in these experiments were obtained from a dealer and were not bred in our own laboratory. It is possible that the accuracy of our results would have been improved if we had been able to use a constant strain of animals kept under more carefully controlled conditions.

The specificity of this modified adrenal ascorbic acid depletion technique for the bioassay of ACTH has not been fully examined. It was found that intravenous injections of heparinized, freshly shed rats' blood produced no fall in adrenal ascorbic acid concentration. There still existed the possibility that toxic impurities in the samples of ACTH injected could be responsible for adrenal ascorbic acid depletion. To examine this possibility we mixed rats' blood with an equal volume of normal saline containing 5 μg. ACTH per ml. The mixture was incubated at 37°C. for three hours. Since Reiss, Badrick, Halkerston and Plaice (1951) had reported the extreme instability of ACTH in blood *in vitro*, it was believed that this treatment would cause complete inactivation of the ACTH. This was found to be true, and when the incubated mixture was injected intravenously into rats pre-treated with DCA, no adrenal ascorbic acid depletion occurred. It was reasonable to assume that most of the

with ether and unilaterally adrenalectomized on the left side. One hour later the animals were killed and their right adrenal glands were removed. It was found that treatment of the rats with 15 mg. DCA, four hours before unilateral adrenalectomy, completely prevented the adrenal ascorbic acid depletion which normally followed the operation. Smaller doses of DCA were only partially effective in inhibiting the increased pituitary adrenocorticotrophic activity caused by this form of stress.

While it is generally agreed that the adrenocorticotrophic activity of the pituitary gland is controlled by a peripheral humoral mechanism, there is some confusion in the literature concerning the ability of DCA to depress the increased secretion of ACTH caused by stress. Sayers and Sayers (1947) found that pre-treatment of rats with small doses of DCA prevented the adrenal ascorbic acid depletion caused by exposure of the animals to cold. On the other hand Moya and Selye (1948), Gershberg, Fry, Brobeck and Long (1950) and Hall, Finerty, Hall and Hess (1951) have been unable to show that DCA tended to prevent the adrenal ascorbic acid depletion caused by subjection of rats to stress.

Our experiments indicate that DCA is effective in preventing pituitary adrenocorticotrophic activity, provided that it is administered in sufficiently large doses. It is doubtful whether our results augment the evidence that the rate of secretion of ACTH by the adenohypophysis is controlled by a peripheral-humoral mechanism; they have shown that pituitary adrenocorticotrophic activity can be prevented by the previous treatment of rats with DCA, but the doses of the steroid were so great that the physiological significance of the results is obscure.

Having established the fact that pre-treatment of rats with 15 mg. DCA could prevent completely the adrenal ascorbic acid depletion caused by unilateral adrenalectomy, we proceeded to study the effect of intravenous injections of ACTH into rats in which pituitary adrenocorticotrophic activity had been blocked in this way.

It was found that control injections of normal saline produced no adrenal ascorbic acid depletion in DCA-treated rats. Intravenous ACTH caused a fall in the adrenal ascorbic acid concentration. Over a range of doses of ACTH equivalent to 0·08 μg. to 1·25 μg. International Standard, there existed a linear relationship between the logarithm of the dose and the adrenal ascorbic acid depletion. Doses of ACTH greater than 1·25 μg. produced no larger responses.

The mean value obtained for the index of precision of the method (λ=0·463) was similar to that obtained in our own laboratory using the Sayers' method for the bioassay of ACTH, and that reported by Greenspan, Li, Simpson and Evans (1950) using hypophysectomized animals. We have been unable to obtain the precision which many other workers, using the Sayers' method, have reported. However, all the animals used in these experiments were obtained from a dealer and were not bred in our own laboratory. It is possible that the accuracy of our results would have been improved if we had been able to use a constant strain of animals kept under more carefully controlled conditions.

The specificity of this modified adrenal ascorbic acid depletion technique for the bioassay of ACTH has not been fully examined. It was found that intravenous injections of heparinized, freshly shed rats' blood produced no fall in adrenal ascorbic acid concentration. There still existed the possibility that toxic impurities in the samples of ACTH injected could be responsible for adrenal ascorbic acid depletion. To examine this possibility we mixed rats' blood with an equal volume of normal saline containing 5 μg. ACTH per ml. The mixture was incubated at 37°C for three hours. Since Reiss, Badrick, Halkerston and Plaice (1951) had reported the extreme instability of ACTH in blood *in vitro*, it was believed that this treatment would cause complete inactivation of the ACTH. This was found to be true, and when the incubated mixture was injected intravenously into rats pre-treated with DCA, no adrenal ascorbic acid depletion occurred. It was reasonable to assume that most of the

with ether and unilaterally adrenalectomized on the left side. One hour later the animals were killed and their right adrenal glands were removed. It was found that treatment of the rats with 15 mg. DCA, four hours before unilateral adrenalectomy, completely prevented the adrenal ascorbic acid depletion which normally followed the operation. Smaller doses of DCA were only partially effective in inhibiting the increased pituitary adrenocorticotrophic activity caused by this form of stress.

While it is generally agreed that the adrenocorticotrophic activity of the pituitary gland is controlled by a peripheral humoral mechanism, there is some confusion in the literature concerning the ability of DCA to depress the increased secretion of ACTH caused by stress. Sayers and Sayers (1947) found that pre-treatment of rats with small doses of DCA prevented the adrenal ascorbic acid depletion caused by exposure of the animals to cold. On the other hand Moya and Selye (1948), Gershberg, Fry, Brobeck and Long (1950) and Hall, Finerty, Hall and Hess (1951) have been unable to show that DCA tended to prevent the adrenal ascorbic acid depletion caused by subjection of rats to stress.

Our experiments indicate that DCA is effective in preventing pituitary adrenocorticotrophic activity, provided that it is administered in sufficiently large doses. It is doubtful whether our results augment the evidence that the rate of secretion of ACTH by the adenohypophysis is controlled by a peripheral-humoral mechanism; they have shown that pituitary adrenocorticotrophic activity can be prevented by the previous treatment of rats with DCA, but the doses of the steroid were so great that the physiological significance of the results is obscure.

Having established the fact that pre-treatment of rats with 15 mg. DCA could prevent completely the adrenal ascorbic acid depletion caused by unilateral adrenalectomy, we proceeded to study the effect of intravenous injections of ACTH into rats in which pituitary adrenocorticotrophic activity had been blocked in this way.

NELSON Have you tried giving cortisone orally rather than by injection?

BUTTLE: No. Would you be able to obtain a sufficiently high blood level in this way?

[illegible]

ACTH assay, because the sensitivity and the slope of the curve were not satisfactory.

HODGES: Our slope was steep and the method was very sensitive Did you get complete pituitary block with DCA?

H J. ROBINSON: No. I think you'll find that adrenal cortical extract works better.

HODGES: We're going to try that next.

HARRIS Isn't that all a quantitative difference? I think that Dr. Sayers found that nothing would block the effect of sufficient stress.

HODGES: Our results are different from those of Sayers and Sayers. The doses of DCA required to prevent the increased secretion of ACTH, in response to the stress of ether anæsthesia and unilateral adrenalectomy, were fifty to one hundred times as great as those which Sayers and Sayers found necessary to prevent the effect of cold Sayers and Sayers reported that the adrenal ascorbic acid depletion, caused by cold (4°C. for 1 hour) could be prevented by previous treatment of the rats with only 200 μg. DCA (per 100 g. body weight), injected subcutaneously in oil immediately before the application of the stress In our experience, doses of this order have produced no inhibition of pituitary adrenocorticotrophic activity.

P. MORRIS: What were your mean control values? Roughly 400?

HODGES. About 400–420 mg ascorbic acid per 100 g adrenal tissue.

P. MORRIS: And what was the range of your control values?

HODGES: Using groups of about twelve animals our standard error was generally between ± 10 and ± 20. We do not possess a colony of rats and all the animals were obtained from dealers. The scatter of our observations was wide, the results were disappointing

[illegible]

not be explained other than by some form of depletion Some were only 25 per cent of the others.

FINGL: The maximum adrenal ascorbic acid depletion by adrenocorticotrophic hormone in your DCA-blocked animals was about 120 mg./100 g. gland. How does that value compare with the maximal depletion in hypophysectomized animals?

HODGES: It's not as great We get a maximal depletion of about 160

impurities in the ACTH were unaffected by this treatment. It was considered, therefore, that the adrenal ascorbic acid depletion, seen in attempts to assay ACTH in rats pre-treated with DCA, was entirely due to the adrenocorticotrophic activity of the samples under test.

Although the method has not provided us with accurate results, we consider it to be worthy of further examination. It is more easily carried out than the Sayers' method and more animals may be used conveniently in the performance of the test since the need for hypophysectomy is obviated. There seems to be no theoretical reason why the technique should not ultimately prove as satisfactory as the Sayers' method, provided that the inhibition of pituitary adrenocorticotrophic activity is effective, and the mobilization of endogenous ACTH is completely prevented.

REFERENCES

Gershberg, H., Fry, E. G., Brobeck, J. R., and Long, C. N. H. (1950). *Yale J. Biol Med.*, 23, 32.
Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M. (1950). In *Hormone Assay* (ed. C. W. Emmens), p. 205. New York: Acad. Press, Inc
Hall, C. E., Finerty, J. C., Hall, O., and Hess, M. (1951). *Endocrinology*, 48, 591.
Long, C. N. H (1947). *Fed. Proc.*, 6, 461.
Moya, F., and Selye, H. (1948). *Proc. Soc. exp. Biol. N.Y.*, 68, 529
Reiss, M., Badrick, F. E., Halkerston, I. D. K., and Plaice, C. (1951). *Nature, Lond.*, 168, 206
Sayers, G., and Sayers, M A. (1947) *Endocrinology*, 40, 265.
Sayers, M. A., Sayers, G., and Woodbury, L. A. (1948). *Endocrinology*, 42, 379.

DISCUSSION

Buttle: We also investigated the use of cortisone in our attempts to obtain inhibition of pituitary adrenocorticotrophic activity. It was found that the intraperitoneal administration of cortisone acetate sus- [illegible] This

THE MEASUREMENT OF ACTH ACTIVITY IN PLASMA

C. H. GRAY and D. M V PARROTT

For some time we have been interested in the study of the rôle of the adrenocorticotrophic hormone in various pathological conditions. The determination of ACTH in plasma satisfied our requirements in this regard, as well as being of more general interest in demonstrating the link between the anterior pituitary and the adrenal cortex. Much of what we have to say has already been communicated to the Society for Endocrinology, but as we understand that some workers have difficulty in demonstrating the presence of ACTH in plasma, a summary of our work in this field may provide the basis of a useful and helpful discussion.

The earlier experiments alleged to show the presence of ACTH in plasma or serum were performed on intact mice which had received no pre-treatment with deoxycorticosterone acetate as described in the previous paper (p 147) The injected materials were active in increasing the weight of the adrenals or in altering the histology of the cortex These results must be disregarded since they may have been due to the injected substance stimulating the pituitary of the assay animal to produce endogenous ACTH Indeed, our own work has shown it unlikely that any ACTH activity would remain after the time necessary for separation of serum.

The introduction by Sayers, Sayers and Woodbury (1948) of the ascorbic acid depletion method of assay of ACTH was an important advance in this problem, for it is much more sensitive than any assay previously used. Using this method Cooke, Graetzer and Reiss (1948) demonstrated the activity of material precipitated by acetone from normal human plasma. Taylor, Albert and Sprague (1949) obtained negative

mg. ascorbic acid per 100 g. adrenal tissue in hypophysectomized animals. The control adrenal ascorbic acid level is not as high in DCA-treated rats as in hypophysectomized animals twenty-four hours after the operation. Thus the mean value for the control adrenal ascorbic acid level in our modified assay is lower than the control level in the Sayers

the reason

agine that

the peri-

toneum.

HODGES We intend repeating the experiments, administering the DCA subcutaneously

VOGT. Of course it may not help because absorption may be slow then, but on the other hand you may be able to get away with smaller doses.

subcutaneous adminis-

animals are very expensive and one would really like to use normal animals.

I would like to call your attention to the fact that we have been able to modify the ascorbic acid assay method, shortening the incubation time from 3 hours incubation to 45 minutes, merely by raising the temperature to 57°C. This method appeared in *Acta Endocrinologica*, 1951, 8, 247, in a paper by Geschwind, Williams and myself.

results and the large amount of protein in the final precipitate often proved fatal when injected into the assay rats. Moreover, it was impossible to recover more than a negligible amount of ACTH added to blood even if the blood were immediately extracted. The large bulk of protein appears to adsorb added ACTH or impede its extraction. We have therefore abandoned attempts to determine ACTH in whole blood.

It appeared important to examine the effect of varying the time and method of extraction on the percentage recovery of ACTH added to plasma.

Assay Procedure

We have used the assay procedure exactly as described by Sayers, Sayers and Woodbury (1948) except that female Wistar rats, weighing 140–160 g. were used instead of Sprague Dawley male rats. Although male rats are slightly more sensitive, those of our own colony were required for other assays. However, the adrenals of male rats are smaller, weighing on an average 15 mg. as against 25 mg. in the females, so that there is a greater error in weighing and estimation. An average of 38 animals in 3–4 doses have been used for the calculation of each regression line. The slope *b* was checked at frequent intervals. Sixteen animals were usually used for each plasma assay: eight for plasma and eight for a single dose of ACTH to determine the value of the constant *a* of the regression line. We have found the slope *b* to be relatively unchanged over a period of 12 months (Table I)

First, the recovery was investigated when Armour ACTH was subjected to Bornstein and Trewhella's procedure.

Table I

Sex	*Date*	*b*	*s*	*λ*
F	February–March 1951	136 46	30·95	0 227
F	August–November 1951	133 86	21·40	0 160
F	December 1951–March 1952	112 81	22 18	0 197
M	December 1951–March 1952	140 86	38 88	0 276

results with untreated normal human sera although sera from cases of Addison's disease were active. Equivocal results were obtained with sera from Cushing's syndrome Bornstein and Trewhella (1950), also using Sayers' assay, obtained an active fraction from an acid acetone extract of normal plasma. High levels of activity were found in plasma from cases of Cushing's syndrome and from patients under the severe stress of surgical operation or cardiac failure, whereas negligible activity was obtained in the material obtained from a case of Simmonds' disease. We have confirmed that the method of extraction described by Bornstein and Trewhella, which is based upon that originally described by Lyons (1937), does yield material significantly active in the Sayers' assay.

It appeared of interest to investigate the activity of the acid-acetone extracts of plasma by some method other than the indirect adrenal ascorbic acid depletion method. In collaboration with Bornstein we investigated the activity of acid-acetone extracts by the adrenal maintenance test of Simpson, Evans and Li (1943). The results, which have already been published (Bornstein, Gray and Parrott, 1952) showed a statistically significant increase of adrenal weight of injected hypophysectomized rats over control animals. Histological examination of the adrenals showed a partial restoration of the cortex in the injected animals.

We were therefore convinced of the possibility of demonstrating ACTH activity in plasma and we have attempted to improve the extraction procedure. There is rapid inactivation of any ACTH present if there is delay between blood collection and treatment of the plasma with acid acetone. Reiss, Badrick, Halkerston and Plaice (1951) have observed a similar rapid loss of adrenocorticotrophic activity of a pituitary preparation when incubated with heparinized plasma from rats, rabbits and humans.

Since there is an inevitable delay in separation of plasma from red cells, we have attempted to prepare active extracts from whole blood. However, there was poor duplication of

for five minutes (it is unnecessary to allow the extract to stand overnight at 4°C. as described in the procedure of Bornstein and Trewhella) the resultant precipitate is centrifuged and dried. With this method, the range of plasma ACTH for normal females is slightly higher than that obtained with the original method (Table III). We have found no advantage in the double extraction procedure described by Gemzell, Van Dyke, Tobias and Evans (1951).

Table III

PLASMA ACTH (μG. PER 100 ML PLASMA) OF NORMAL FEMALES AGED 21–30 YEARS

	No	Range	Mean	S E
Old method	6	41–86	56	6 73
New method	5	60–111	83	6 77

Although there is loss of ACTH activity when there is delay in separation of plasma, with proper precautions this loss need not be so rapid as we had previously believed. The loss of activity may be considerably reduced by collection into chilled vessels and immediate separation and extraction of plasma.

Normal plasma has low ACTH activity, and since readings of the plasma extract alone would fall on the lower and least reliable part of the standard curve, we have found it advantageous to add 0·5 or 1·0 μg. of standard ACTH to bring the level of the readings to the linear part of the curve, i.e. between 0 5–2·0 μg There was no significant difference between the results obtained whether ACTH was added to plasma immediately after separation and before extraction, or to the solution of the final precipitate immediately before assay. This latter course is more convenient and is usually followed.

Plasma from six cases of Cushing's syndrome has been estimated for ACTH activity; five of them gave values (120–310 μg ACTH/100 ml. plasma) above normal limits,

2 mg. quantities of LA-1-A ACTH in N/50 acetic acid were extracted with acid acetone for varying periods of time up to four hours. The ACTH was completely soluble but was precipitated on increasing the acetone concentration. There was no significant difference between the ACTH activity of the original material and that treated with acid acetone in this way. When ACTH was added to plasma good recovery was obtained if the mixture was immediately extracted and centrifuged (Table II). When the acid acetone was allowed

Table II

RECOVERY OF ARMOUR ACTH ADDED TO PLASMA

Dose	*Time of extraction*	*Mean fall of adrenal ascorbic acid mg per 100 g* *
2 8 μg Armour ACTH+0 45 ml. plasma/100 g body weight	0 hr.	114±19†
ditto	1 hr	137±10
2 8 μg. Armour ACTH		111±24

For group plasma (0 hr.) and plasma (1 hr)
$t=2\ 73$ where $n=9$, $p=0\ 05$–$0\ 02$
For group plasma (1 hr.) and standard ACTH solution
$t=2\ 4$ where $n=11$, $p=0\ 05$–$0\ 02$

*6–8 animals per group
†Standard deviation

to extract the precipitate for one hour, recoveries of ACTH were usually greater than 100 per cent and appeared to be due to extraction of endogenous ACTH in the plasma However, this higher recovery did not always take place and, when it did not, 2½ hours extraction resulted in a 60 per cent loss in activity. It appeared, therefore, that the plasma ACTH might be more effectively extracted by a shorter and more vigorous procedure, and the following modifications have been adopted. Plasma is extracted with four volumes of acid acetone (containing 2·5 per cent v/v conc. HCl) by grinding the mixture with sand in a mortar and pestle for five minutes. The precipitate is centrifuged and 40 ml. of acetone added to each 10 ml. of supernatant. After standing

Stack-Dunne: Do you collect blood from your normal humans first thing in the morning or in the afternoon?

Parrott: The usual time is about half-past ten to eleven in the morning.

th

la

patient?

Parrott: I've done two separate estimations on the same patients, but I've never carried out a series of estimations. The only person I've done many times is myself, and I stay relatively constant

Buttle: Didn't Bornstein find a difference in a patient before and after operation? He did a number of estimations in one patient, didn't he? Have you tried to repeat that?

Gray. No.

Cater It might be interesting to measure a number of normal people under basal conditions.

Loraine. Have you employed purification with low temperature centrifugation?

Parrott: We haven't got a centrifuge that we can keep at low temperature. I keep the blood as cold as I can—collect it as rapidly as possible, put into centrifuge tubes and then spin as rapidly and take off as quickly as I can.

Gaddum: How quickly is it inactivated if you take no precautions?

Parrott: I haven't any definite time curve on that, but in one estimation, on leaving whole blood to stand, there was a loss of 40 per cent of activity within 10 min I really expected it to be more rapid than that, but I think that in some of our other observations when we thought it was very rapidly destroyed, it was perhaps due to inactivation in some other way

Cope Have you tried whether you could stop inactivation in a siliconed vessel?

Gray: No.

Bayliss With regard to the values that were higher than you expected, I calculate that you must have about 2 5 mg in the total blood that's circulating (about 4–5 l of blood, and 100 μg per 100 ml of plasma) which is quite a lot, when you think of the effect you get with an intravenous injection of 5 mg of ACTH over a 24-hour period

Harris. I wonder whether the method of collecting blood would stress some of the patients Do you think that that might produce a rise in the ACTH level?

Parrott. It could with a nervous patient

Gray. Most of the normals and the patients were used to being bled.

Harris Do you see any change in the level in particular patients over the time they are becoming used to the procedure?

Parrott. No.

Fraser Have you assayed anyone receiving cortisone?

the highest activity being found in a case with pituitary tumour. There was no significant change in plasma ACTH of two cases following ⅞ unilateral adrenalectomy but a more dramatic result may follow removal of a second adrenal.

The fiducial limits of error have been calculated for assays of 15 different plasma extracts, each with 0·5 or 1 µg. of added ACTH. The fiducial lower limits exceed the amount of added ACTH in all except two instances. We therefore feel justified in assuming that a measurement of plasma ACTH is possible, but can only be regarded at present as semi-quantitative.

REFERENCES

BORNSTEIN, J., GRAY, C. H., and PARROTT, D. M. V. (1952) *J. Endocrinol.*, 8, 40.

BORNSTEIN, J., and TREWHELLA, P. (1950). *Lancet*, ii, 678.

COOKE, D. S., GRAETZER, E., and REISS, M. (1948) *J. Endocrinol.*, 5, lxxxix.

GEMZELL, C. A., VAN DYKE, D. C., TOBIAS, C. A., and EVANS, H. M. (1951) *Endocrinology*, 49, 325.

LYONS, W. R. (1937). *Proc. Soc. exp. Biol., N.Y.*, 35, 645.

REISS, M., BADRICK, F. E., HALKERSTON, I. D. K., and PLAICE, C. (1951). *Nature, Lond.*, 168, 206.

SAYERS, M. A., SAYERS, G., and WOODBURY, L. A. (1948). *Endocrinology*, 42, 379.

SIMPSON, M. E., EVANS, H. M., and LI, C. H. (1943) *Endocrinology*, 33, 261.

TAYLOR, A. B., ALBERT, A., and SPRAGUE, R. G. (1949). *Endocrinology*, 45, 335.

DISCUSSION

ting?

, unextracted.

ct one cc. without

any damage.

STACK-DUNNE. Do you collect blood from your normal humans first thing in the morning or in the afternoon?

PARROTT: The usual time is about half-past ten to eleven in the morning.

[illegible] patient?

PARROTT: I've done two separate estimations on the same patients, but I've never carried out a series of estimations The only person I've done many times is myself, and I stay relatively constant

BUTTLE: Didn't Bornstein find a difference in a patient before and after operation? He did a number of estimations in one patient, didn't he? Have you tried to repeat that?

GRAY: No

CATER: It might be interesting to measure a number of normal people under basal conditions.

LORAINE: Have you employed purification with low temperature centrifugation?

PARROTT. We haven't got a centrifuge that we can keep at low temperature. I keep the blood as cold as I can—collect it as rapidly as possible, put into centrifuge tubes and then spin as rapidly and take off as quickly as I can.

GADDUM How quickly is it inactivated if you take no precautions?

PARROTT I haven't any definite time curve on that, but in one estimation, on leaving whole blood to stand, there was a loss of 40 per cent of activity within 10 min I really expected it to be more rapid than that, but I think that in some of our other observations when we thought it was very rapidly destroyed, it was perhaps due to inactivation in some other way

COPE: Have you tried whether you could stop inactivation in a siliconed vessel?

GRAY. No

BAYLISS With regard to the values that were higher than you expected, I calculate that you must have about 2 5 mg in the total blood that's circulating (about 4–5 l of blood, and 100 μg per 100 ml. of plasma) which is quite a lot, when you think of the effect you get with an intravenous injection of 5 mg of ACTH over a 24-hour period

HARRIS. I wonder whether the method of collecting blood would stress some of the patients Do you think that that might produce a rise in the ACTH level?

PARROTT It could with a nervous patient

GRAY Most of the normals and the patients were used to being bled.

HARRIS Do you see any change in the level in particular patients over the time they are becoming used to the procedure?

PARROTT: No

FRASER: Have you assayed anyone receiving cortisone?

GRAY: No.

C. J. O. R. MORRIS: It would be interesting to try acidifying the blood or plasma. It might be much more stable then and easier to work up. That would be in agreement with the known properties of ACTH.

P. MORRIS. Have you tried any estimations on urine at all?

GRAY: No.

again and it gave the largest depletion we have ever had. We did that about four times, and we had a high, a negative, and two intermediate values. In virilism, the values were completely scattered, and all assays were done in groups where we had an arbitrary standard In normal urine we didn't get any significant depletions. In four rheumatoid arthritics there was definitely a small depletion, but since it was on the verge of significance it would require a very large number of animals to establish the effect.

GRAY. How many animals per group?

P. MORRIS: Four And in the case of Addison's, six. The urine was collected in a winchester containing about 10 ml. of glacial acetic acid, and that was left until the next morning.

GADDUM: You extract it?

... body weight

... rial

... irly

an assay o...

his urine. ...

the same.

... to become lower in plasma

... think

... these

... 't he?

PARROTT. He found an enzyme in blood which was capable of destroying ACTH ...

enzyme.

H. J. ROBINSON: I believe Dr. Pincus also feels that the rate of destruction of ACTH is different in plasma of various animal species.

LORAINE: Have you any information on the ACTH content of placentæ? I think Dr. Opsahl said she was able to extract ACTH from the placenta using a method depending on hyaluronidase inhibition in mice.

GRAY: We have no information.

MACLAGAN. Can you tell us if this has been looked for in cerebral spinal fluid?

GRAY: I don't know if it has. We haven't investigated that

GRAY: No.

C. J. O. R. MORRIS: It would be interesting to try acidifying the blood or plasma. It might be much more stable then and easier to work up. That would be in agreement with the known properties of ACTH.

P. MORRIS: Have you tried any estimations on urine at all?

GRAY: No.

SEGALOFF: We tried urine quite extensively, and in two of our patients with Cushing's disease were able to get amounts that were positive in the Sayers test, but we've never gotten it in any other urine.

P. MORRIS: We have found an ascorbic acid depleting factor in urine in rheumatoid arthritics before ACTH. It was in the lower range. We did the estimation in untreated Addison's; it was negative. We did it again and it gave the largest depletion we have ever had. We did that about four times, and we had a high, a negative, and two intermediate values. In virilism, the values were completely scattered, and all assays were done in groups where we had an arbitrary standard. In normal urine we didn't get any significant depletions. In four rheumatoid arthritics there was definitely a small depletion, but since it was on the verge of significance it would require a very large number of animals to establish the effect.

GRAY: How many animals per group?

P. MORRIS: Four. And in the case of Addison's, six. The urine was collected in a winchester containing about 10 ml. of glacial acetic acid, and that was left until the next morning.

GADDUM: You extract it?

P. MORRIS: No. Just inject it neat. 1 ml/100 g. body weight.

CROOKE: Would your data support the contention that the material is inactivated within 10 min. in plasma?

P. MORRIS: We haven't any data on that. However, I think it's fairly stable in urine under acid conditions at 0°C., because I had to repeat an assay on a nephritic that was having ACTH. He excreted ACTH in his urine. We kept the urine and repeated it, and the value was roughly the same.

C. J. O. R. MORRIS: You would expect it to become lower in plasma than in urine, probably due to the proteolytic enzymes. And I think that at about *p*H 2 one would expect a higher stability, because these proteases generally seem to have *p*H optima around 4 and 8.

GRAY: I think Pincus has some information on that point, hasn't he? I don't know if he published it.

PARROTT: He found an enzyme in blood which was capable of destroying ACTH.

SEGALOFF: As I understand Dr. Pincus's latest view, some of the preparations of ACTH have what he considers a co-enzyme. Some ACTH preparations are stable when incubated in serum and some of them are not, and he thinks that it is due to the presence or absence of this co-enzyme.

H. J. ROBINSON: I believe Dr. Pincus also feels that the rate of destruction of ACTH is different in plasma of various animal species.

leucocytes and total white cell count is often delayed to 8–10 hours following the administration of the steroid

The effects of free compound F rather than the acetate are shown in Fig. 2. The rise and fall of blood hormone levels are somewhat more rapid than when the acetate of the compound is given. This may well be explained on the basis of

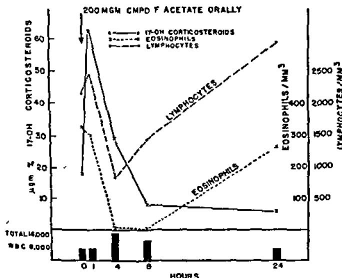


FIG. 1. Blood levels of 17-hydroxycorticosteroids and leucocytes following oral administration of Compound F acetate (Nelson, Sandberg, Palmer and Tyler, 1952.)

increased solubility of the free steroids in physiological solutions.

In a number of cases to whom 200 mg. of Compounds E or F was administered the steroid blood levels have fallen below normal at the 8–24 hour periods This was interpreted as representing a compensatory decreased endogenous production following tissue saturation by the large doses of steroid administered.

EOSINOPHILS, LYMPHOCYTES, AND 17-HYDROXYCORTICOSTEROIDS AS INDICES OF ADRENOCORTICAL ACTIVITY

D H NELSON

MEASUREMENTS of eosinophils (Hills, Forsham and Finch, 1948) and lymphocytes (Dougherty and White, 1943) in peripheral blood have been employed as indices of adrenocortical activity. The development of a method for the measurement of 17-hydroxycorticosteroids in blood (Nelson and Samuels, 1952) has made possible the correlation of changes in absolute numbers of circulating eosinophils and lymphocytes with blood levels of adrenal steroids. Changes in circulating blood elements have been measured simultaneously with the administration of 17-hydroxycorticosterone, 17-hydroxy-11-dehydrocorticosterone, ACTH, and epinephrine.

Response of 17-Hydroxycorticosteroids, Eosinophils, and Lymphocytes to Oral Administration of the Free or Acetate Forms of Compounds E and F

Following the oral administration of 200 mg. Compound F acetate there is an immediate rise in blood levels of 17-hydroxycorticosteroids, as is illustrated in Fig. 1. Peak levels of the steroid are found in the circulation approximately one hour following ingestion of the compound and levels have returned to or below normal in eight hours.

An occasional short initial rise is followed by a decrease in both types of leucocytes to a minimum at 4–6 hours. It can be seen that although the peak level of steroid has been passed after about one hour the total eosinophil and lymphocyte counts continue to decrease for some time thereafter.

The time relationships are very similar for the eosinophils and lymphocytes, but the peak rise in the polymorphonuclear

Similar effects to those described for compound F were found for Compound E. The only demonstrable difference in action between the two compounds was the greater increase in polymorphonuclear leucocytes after Compound F was given to the subject.

The relative quantitative relations of these indices following varying doses of Compound F acetate given orally to a single subject are shown in Fig. 3. Although there is not a direct quantitative relationship between the dose of steroid administered and the fall in eosinophils there does appear to be a rough correlation. The eosinophil levels are also observed to stay depressed longer as the steroid levels are maintained by larger doses of hormone.

Response to Free or Acetate Forms of Compound E and F given Intramuscularly

The effect of 200 mg. free Compound E given to a normal subject intramuscularly is illustrated in Fig. 4. As was typical of other similar studies, there was no effect on blood

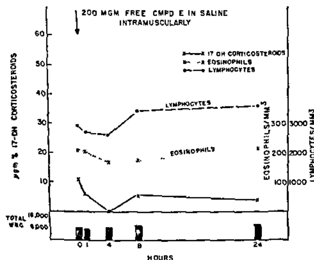


Fig. 4. One type of response to the administration of free Compound E intramuscularly

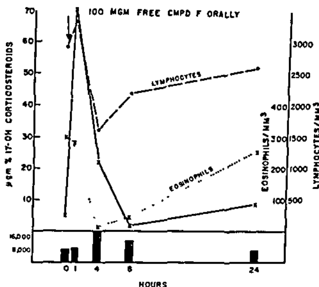


FIG. 2 Blood levels of 17-hydroxycorticosteroids and leucocytes following oral administration of free Compound F.

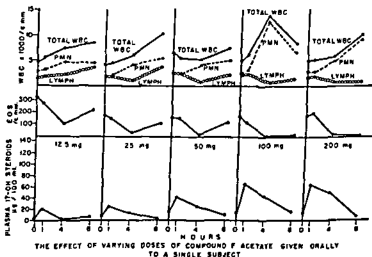


FIG. 3 The effect of varying doses of Compound F acetate given orally to the same subject. (Nelson, Sandberg, Palmer and Tyler, 1952.)

(Nelson, Samuels, Willardson and Tyler, 1951). The correlation of this rise in 17-hydroxycorticosteroids with the blood white cells is shown in Fig. 6. Relatively small changes in corticoid levels are seen to cause a very significant change in the quantity of these circulating cells. The effect is a transitory one, however, with a return to normal levels demonstrable in eight hours.

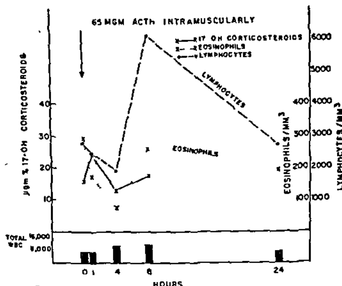


FIG. 6. Response of blood 17-hydroxycorticosteroids to intramuscular ACTH, and subsequent changes in circulating leucocytes.

Continuous intravenously administered ACTH results in markedly elevated corticosteroid blood levels (Ingle, 1950, Nelson, Samuels, Willardson and Tyler, 1951) Fifteen to 30 mg. of ACTH given intramuscularly or subcutaneously every six hours resulted in small unsustained rises in steroid levels. As little as 5 mg. of ACTH given in a continuous infusion over 24 hours produced very high steroid levels which

17-hydroxycorticosteroid levels nor significant variation i the circulating leucocytes. *In two out of four cases give* Compound E acetate intramuscularly there was a significan rise in blood steroid levels which was prolonged over a perio of 24 hours in one case, but no rise was seen in five case given 200 mg. Compound F acetate i.m.

Significant effects were obtained when 200 mg. free Com-

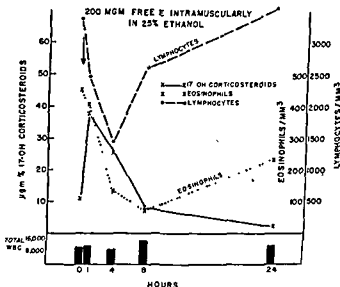


FIG. 5. Response of blood 17-hydroxycorticosteroids and leucocytes to free Compound E given intramuscularly in 25 per cent ethanol.

pound E was given i.m. in 25 per cent ethanol solution (Fig. 5). This would suggest that the lack of response often seen when the steroids are given intramuscularly is due to lack of absorption, and the administration in ethanol solution was a means of increasing absorption from the injection site.

Response to ACTH

When ACTH is given intramuscularly to a normal man the peak rise in steroid level is seen at 1–2 hours after injection

circulation. Whether this represents increased tissue utilization of the adrenal steroids in association with epinephrine action is yet to be determined.

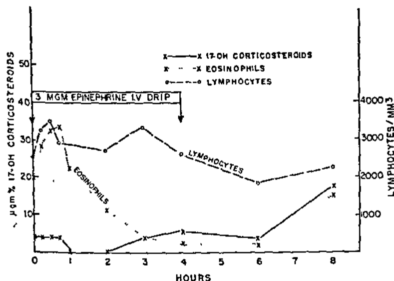


FIG 7. Response of blood 17-hydroxycorticosteroids and leucocytes to intravenously administered epinephrine

Summary

The marked response of blood eosinophils, lymphocytes, and neutrophils to large doses of Compound E or F has been correlated with blood levels of these hormones. ACTH administration also brings about good cellular response in association with relatively small increases in blood levels of 17-hydroxycorticosteroids. The four-hour maximum fall observed with eosinophils and lymphocytes is seen to be a delayed response to elevations in blood steroids, which are usually maximum at least three hours prior to the maximum effect on the leucocytes.

Oral administration of either free or acetate forms of these steroids is highly effective in raising blood levels of the compounds but intramuscular administration gives quite variable

were maintained throughout the period of administration. The blood levels of adrenal steroid returned rapidly toward normal when the ACTH infusion was discontinued. Within four hours, the 17-hydroxycorticosteroid level had returned almost to the initial levels.

Preliminary studies carried out with Dr. Bliss of the Department of Psychiatry indicate that there are some dosage response differences when single 5 or 15 mg. doses of ACTH are given intravenously to normal subjects. The exact quantitative aspects of this work are not yet completed.

Response to Epinephrine

A certain amount of confusion exists in the literature in regard to the relation of epinephrine to the leucocytes and to adrenal cortical steroid production. Recant *et al.* (1950) demonstrated the marked effect on eosinophils seen when epinephrine is given intramuscularly or intravenously to normal subjects This effect could be prevented by adrenalectomy or hypophysectomy in rats. Similar effects have been noted by other workers using adrenal ascorbic acid and cholesterol as the means of indicating adrenal activity (Long and Fry, 1945). Vogt (1944) was able to demonstrate an increase in steroids secreted into the adrenal vein when epinephrine was given to dogs. The decreased eosinophil response seen in hypophysectomized animals is suggestive evidence that the effect of epinephrine is at least partly due to adrenal secretion as mediated by ACTH release from the hypophysis.

The effects of 3 mg. epinephrine given intravenously to a normal subject over a period of four hours is illustrated in Fig. 7. Although there is a definite decrease in eosinophils and a significant rise in neutrophils and total white blood count, there was no demonstrable rise in 17-hydroxycorticosteroids in the peripheral blood stream. It would appear from these data that if the adrenal gland is secreting increased quantities of steroids following the stimulus with epinephrine, that these steroids are being rapidly removed from the

cases took all the fractions and measured them We wondered whether we could extract the acetate if it were circulating in the blood, and found that when the acetate is given intravenously we can measure very nice levels of the compound circulating in the blood stream. There's some question as to whether the effects on the leucocytes are quite as good as one would expect from giving the free compound.

BUSH: I wonder whether your lack of effect with the free compound on circulating eosinophils and lymphocytes, and also your rather smaller chemical effects, might be due to a change in esterase activity at the injection site, whether you were in fact getting absorption of the acetate by diffusion plus a small amount of free compound after hydrolysis, or absorption of the free compound only.

NELSON: I think that is a possibility, that the acetate has to be split off before it exerts its full action. I think our findings when the E acetate was given demonstrate some individual differences, in that half the cases do show these marked effects and half of them don't We've also done studies with Compound S free and acetate, and have found no effects with the S.

CATER: Have you any data as to rapidity of destruction in the tissues of the corticosteroids?

to normal within 6–8 hours

VOGT: I was very interested in the case in which you administered

range.

VOGT. Have you ever done an experiment with a smaller dose?

NELSON: Yes, we started out with either 1 or 1 5 mg. of epinephrine, given over the four-hour period, and found no effect, and so we stepped [illegible]

the epinephrine was given

SEGALOFF Incidentally, hasn't Dr Thorn also reported that in his *adrenalectomized* patients the infusion of epinephrine produces a drop in the eosinophils?

results. Some increase in absorption rate of the free compounds can be obtained by administration in ethanol solution.

No rise in blood levels of 17-hydroxycorticosteroids was observed when large doses of epinephrine were given intravenously to a normal subject. There was, however, a marked fall in eosinophils and a moderate decrease in lymphocytes

Acknowledgments

The work described in this paper represents part of collaborative efforts carried out in association with Drs. Leo T. Samuels, Frank H. Tyler, Avery A. Sandberg and Jeffrey Palmer of the Departments of Biochemistry and Medicine, University of Utah College of Medicine The technical assistance of Miss Carma Darley, Mr. Myles E Glenn and Mr. Boyd W. Harding is gratefully acknowledged.

REFERENCES

DOUGHERTY, T F., and WHITE, A. (1943). *Proc. Soc. exp. Biol. Med*, 53, 132.

HILLS, A. G., FORSHAM, P. H, and FINCH, C. A. (1948). *Blood*, 3, 755.

INGLE, D. J. (1950). *J. clin. Endocrinol.*, 10, 1312

LONG, C. N H, and FRY, E G (1945) *Proc. Soc. exp. Biol. Med*, 59, 67.

NELSON, D. H., and SAMUELS, L. T. (1952) *J clin Endocrinol.*, 12, 519.

NELSON, D. H., SAMUELS, L T., WILLARDSON, D. G., and TYLER, F. H (1951) *J. clin. Endocrinol.*, 11, 1021.

NELSON, D. H., SANDBERG, A. A, PALMER, J G., and TYLER, F. H. (1952). *J clin. Invest.*, 31, 843.

RECANT, L., FORSHAM, P. H, and THORN, G. W. (1950). *J. clin. Endocrinol*, 10, 187.

VOGT, M. (1944) *J. Physiol*, 103, 317.

DISCUSSION

BUSH: Have you any other data on the differences between the free compounds and the acetate?

NELSON. In each case now we've given the same dosage of the acetate and free compound to groups of about 6 cases, and the findings have been similar to those shown

BUSH If you're using your usual chromatographic method, I was wondering whether the Compound E acetate might not be hydrolysed when you gave it intramuscularly, and stay as the acetate—then in fact the E acetate in the blood would come out in your front fractions, which I think you usually discard.

NELSON: We considered that possibility, and in a good number of

BUSH. Something that worries me in taking blood samples from patients and trying to correlate cortical steroid concentrations with pathological states, is the question of daily variation Have you any information on whether the normal level is either constant or changes during the day?

NELSON. There is some variation in the cortical steroid level over the day, but the level has never been out of what we call our normal range

BUSH: ±20 per cent and no more?

NELSON. I would say at least that in some individuals. Again there is quite a variation from individual to individual. Some will stay within one or two micrograms over the entire 24-hour period, whereas we have other cases that have varied anywhere from 5 up to 20 μg over the 24-hour period.

ASTWOOD. I was wondering if Dr. Nelson would feel that the intramuscular injection of 25 per cent ethanol would evoke the secretion of adrenocortical steroids?

NELSON: We had control studies on that, and our effects are not due to the injection of ethanol.

CATER Do you think the effect of the alcohol upon increasing the levels in the blood might be due to the fact that it produces an inflammatory reaction in the muscle and therefore enormously increases the rate of absorption?

NELSON: It's a possibility.

CATER: Have you tried any substance which would cause an inflam- [illegible]

[illegible]

BUTT: Have you attempted to measure any bound steroids?

NELSON: We have made some preliminary attempts, and thus far have not been successful in demonstrating any, but we're certainly in no position to say that there aren't some there.

H. J. ROBINSON. It is not quite clear to me why there is a difference in [illegible]

[illegible] doses [illegible] ases of [illegible] ng the [illegible] found [illegible]

NELSON: Yes, I believe so. There is some confusion in the liter as to whether you can get as good a fall of eosinophils You can fall apparently in some adrenalectomized animals and patients rather large doses of epinephrine.

FRASER: Have you given adrenaline together with Compound see whether the change you observed in 17-hydroxysteroids is greater?

NELSON: Yes, we have. But it's quite a problem to decide just to do it Should the cortisone be given first, should they be give gether, or should we give some epinephrine and then some cortis In one study in which an attempt was made to give the two toge we initially obtained much higher levels of steroids in the b stream than we ever had before. A possible explanation for that w be that there was some arteriolar constriction, and that we were

ve were wh

the level fe

will have t

done.

MACLAGAN. May I present some data which I think might hel explain the rises in blood count that Dr. Nelson showed in some o cases. We have been studying the eosinophil counts in intact rab The initial count is about 4,000/mm.[3]. We find with ACTH we g

H. J. ROBINSON If you have not distinguished between the pseu eosinophils and the eosinophils, one cannot possibly draw conclusi regarding the effect of cortisone on the true eosinophils. In the rab the pseudo-eosinophil behaves like a neutrophil and hence cortis does not reduce the number of the cells in the peripheral blood. It l been our experience that the pseudo-eosinophils are actually increa as a result of cortisone therapy whereas the true eosinophils are marke decreased.

MACLAGAN: We haven't yet studied the total white count effe I should have mentioned that there was no rise in temperature af ACTH, but then we know from recent work at the National Institute Medical Research that ACTH tends to abolish the pyrogenic rise temperature anyhow.

HARRIS: We have observed a rise in total white count in this particul species too with ACTH or with stress. This was work with Dr. H. Coll and Dr. J. de Groot.

THE EFFECT OF ADRENAL STEROIDS UPON MUSCLE WORK

DWIGHT J. INGLE

When the gastrocnemius muscle of a normal anæsthetized rat is made to contract by intermittent faradic stimulation, it can sustain a high rate of work output for periods of several days The rat loses its ability to work within a few hours after its adrenal cortices are removed The ability to work can be sustained, almost completely, by the administration of adrenal cortex extracts and certain steroids. This technique has been used as a research tool by the author during the past 20 years. It has also been used as a method for the quantitative bioassay of adrenal cortex extracts and steroids, but more simple procedures are now recommended. The ability of an experimental animal to work over long periods of time may be regarded as a criterion of vigour which has wide applicability in the fields of endocrine physiology and nutrition

History

Addison (1855) listed muscular asthenia as a part of the syndrome of adrenal unsufficiency in man. Albanese (1892) demonstrated experimentally the muscular asthenia of adrenalectomized animals. As confirmed by many other clinical and laboratory investigators, adrenal cortical insufficiency is invariably characterized by an inability to sustain a normal output of work.

During the 1920's, R. G. Hoskins and his associates reported a series of studies on vigour in the rat. As one criterion of vigour, they tested the ability of the gastrocnemius muscle of the anæsthetized rat to respond to faradic stimulation. Among other studies, it was shown that adrenalectomy causes a marked decrease in work output (Gans and Miley, 1927).

much lower than we expected, in fact they were within the normal range. I wonder if this failure of the steroid level in the blood to rise was due to increased utilization by the tissues?

tion, but that's a rather long story

COPE: *One point that is very important is the time factor in these*

ss the

th the

NELSON. *It was the free compound given intravenously.* These differences were much more significant, of course, when we had obtained high levels of the steroid following administration than they were when we were drawing normal samples.

BIBILE: *And would this rate of disappearance in the liver account for the rate of disappearance of your injected compounds?*

NELSON: I'm not sure.

isolated from adrenal cortex extracts and were found to be biologically active in the work test. It was recognized that these steroids did not represent all of the biological activity of adrenal cortex extracts and a search was continued in several laboratories for a more active compound which was expected to replace completely the functional activity of the adrenal glands. In 1937, it seemed that this objective had been realized in the synthesis of 11-deoxycorticosterone (Steiger and Reichstein, 1937). This steroid maintained life and apparent normality in adrenalectomized animals under non-stress conditions at remarkably low dosage.

When, in the fall of 1938, 11-deoxycorticosterone was tested for its effect on work in adrenalectomized rats, it was found to be almost completely ineffective At the same time, it was shown by Dr C. N. H. Long and his associates at Yale University that this compound was without glycogenic activity in the adrenalectomized rat At this time we suspected, erroneously, that the apparent qualitative deficiency of 11-deoxycorticosterone was based upon the slow absorption and utilization of this steroid in acute tests. Subsequent studies proved that the compound was qualitatively deficient in its biological effect upon work and carbohydrate metabolism just as the 11,17-oxygenated steroids were weak in their effect upon electrolyte balance.

The muscle-work test was standardized as a quantitative bioassay procedure (Ingle, 1944) and used in the laboratories of the Upjohn Company to guide research on adrenal cortex extracts as well as the commercial production of adrenal cortex extracts over a period of several years When the more simple liver glycogen deposition test was perfected, its sensitivity and reliability were found to be equal to that of the muscle-work test. Comparisons of most, but not all, adrenal cortex extracts and steroids show a high correlation between the bioassay results of the two tests (Pabst, Sheppard and Kuizenga, 1947). The test for glycogenic activity has now been accepted as a standard procedure in this and other laboratories. The muscle-work test is presently used by us as a research tool.

This series of studies inspired the author and his co-workers, W. M. Hales and W. T. Heron, to initiate, in 1931, a comparative study of vigour in two strains of rats which had been developed by selective breeding on the basis of voluntary activity. It became apparent that methodology was of basic importance in such studies. The total amount of work done by a rat could be made to vary several-fold according to the nature of the stimulus. During the first two years, we were plagued by respiratory failures in our anæsthetized animals. As methodology improved, it became uniformly possible to keep anæsthetized rats working continuously for periods of several days. We were amazed to find that after as much as 10 days of continuous stimulation, the height of muscular contractions of some fed animals exceeded the height of the initial muscular contractions. Anæsthetized fed rats were kept working for periods up to 17 days. Forced work is a severe stress to the animal and work output is a criterion of the adequacy of the animal's adjustment to the stress.

Since my interest in the adrenal cortex was antecedent to a study of muscle-work, it was natural that we should study work performance of the adrenalectomized rat and confirm its lack of vigour. Work performance fell below normal within a few hours following adrenalectomy but this failure could be almost entirely prevented by the administration of extracts of the adrenal cortex (Ingle, 1933). We gained appreciation of the fact that much larger amounts of adrenal cortex extracts are required to sustain the normality of an adrenalectomized animal during a severe stress than are needed to sustain life under non-stress conditions.

In 1933 these studies came to the kind attention of Dr. E. C. Kendall, who supplied adrenal cortex extracts for this research. In 1934, it was my good fortune to join his research group. When cortisone was isolated in 1935 (Mason, Myers and Kendall, 1936), its biological activity was immediately demonstrated in the work-test, whereas it was considered to be inactive by other investigators. Subsequently, 11-dehydrocorticosterone, corticosterone and hydrocortisone were

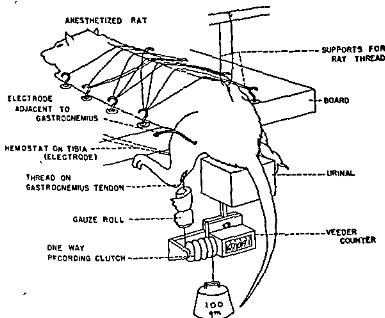


FIG. 1. Diagram of anæsthetized rat prepared for the stimulation of muscle.

Recent and Current Research

When hormones are administered by intermittent injection into the tissues or into one of the body cavities, the quantitative nature of the response may be affected by differences in the physical properties of the hormones which cause differences in rate of absorption and in efficiency of utilization. The continuous intravenous injection of aqueous solutions (usually 5 per cent ethanol) of hormones simulates the manner in which the gland secretes its hormone into the blood more nearly than the procedures of intermittent injection. With this advance in methodology, we have undertaken the study of the two following problems. First, what therapy is necessary to sustain fully the ability of an adrenalectomized rat to work? Second, what is the relative biological potency of individual steroids of the adrenal cortex?

Present Methods

The muscle-work test has gone through a period of evolution which has not yet ended. Over the years we have improved the procedure for stimulating muscle so that the amounts of work elicited from normal rats are much greater than were obtained years ago. We have access to disease-free rats, an essential requisite for the prolonged maintenance of rats under anæsthesia. Different strains of rats vary markedly with respect to their ability to work, and we have access to animals which are vigorous in this respect. Finally, we have achieved an ambition which was conceived years ago, namely, the incorporation of continuous injection equipment with our fatigue apparatus so that hormones and nutrients can be administered by continuous intravenous injection to the working rat.

The details of the muscle-work test have been described (Ingle, 1944). Male rats of the Sprague-Dawley strain are maintained on Archer Dog Pellets until they reach a weight of 200 ± 2 g. The animals are anæsthetized with phenobarbital sodium and cyclopal sodium and are subjected to the stimulation of muscle immediately following removal of the adrenal glands. In most studies the kidneys are also removed. The gastrocnemius muscle of the left hind leg is weighted with 100 g. A Nerve Stimulator, Model B, Upjohn, is used to deliver five pulses per second. The duration of each pulse is 20 milliseconds and the intensity is 20 milliamperes. The distance the weight is lifted is recorded on automatic work adders. Each recorder revolution represents approximately 400 gram-centimeters of work. The animals are enclosed in a temperature cabinet with temperature constant at $28 \pm 0{\cdot}5$°C. The test substances are made up in aqueous solution and are injected into the jugular vein by means of a continuous injection machine. The fluid volume is 20 ml. per 24 hours per rat. By means of this apparatus 12 rats can be studied simultaneously. A diagram of the general procedure is shown in Fig. 1.

organic and inorganic metabolism than any other known compound, it was anticipated that it would be superior to either cortisone or hydrocortisone in the work test. This was not true. Optimal doses of corticosterone had an effect which was approximately equivalent to the effect of optimal doses of the other two compounds and was inferior to optimal doses of extract. The data are illustrated in Fig 3.

None of the single compounds tested in these studies fully substitutes for either the rat's adrenal cortices or for adrenal

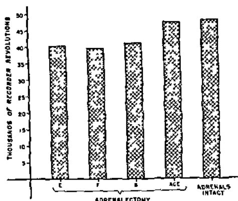


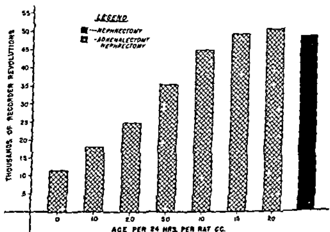
Fig. 3. Total [illegible]

cortex extracts. When cortisone, hydrocortisone and corticosterone are mixed in equal proportions, the peak response is not superior to that elicited by optimal doses of the single compounds When dosage is based upon glycogen units, adrenal cortex extract is superior to equivalent doses of the pure compounds in supporting work (Fig. 4). These results support the hypothesis that adrenal cortex extracts contain one or more unidentified compounds of biological importance.

2. *Comparative Activity of Single Steroids.* Earlier studies (Ingle and Kuizenga, 1945, Pabst, Sheppard and Kuizenga,

1. *Completeness of Replacement Therapy.* As shown by Ingle and Nezamis (1949), the continuous intravenous injection of large amounts of beef adrenal extract can sustain a normal work output in adrenalectomized rats. Doses of 15 to 20 ml. per 24 hours per rat were required. Full replacement has never been achieved by the intermittent injection of adrenal hormones.

In subsequent studies mixed extracts from hog and beef *adrenal glands have been used which contain the biological*



injection of

activity equivalent of 0·1 mg. of hydrocortisone per ml., as assayed by the liver glycogen deposition test. When adrenalectomized-nephrectomized rats were used, it was confirmed that large doses of adrenal cortex extracts do sustain normal or almost normal performance (Fig. 2). Treatment with either cortisone or hydrocortisone failed to sustain a normal output of work (Ingle, Nezamis and Morley (1951*a*). These studies have been extended to corticosterone. Since this steroid represents a better balance between the effects upon

activity of the adrenal cortices of the hypophysectomized rat, especially those fractions which show a dissociation in effects upon adrenal ascorbic acid and adrenal morphology (Ingle and Li, 1952)

In unpublished studies we have seen striking differences in the work performance of rats on different diets, each of which was thought to contain all of the food factors. This criterion of dietary adequacy may deserve exploration. The availability of continuous injection equipment permits systemic studies of intravenous feeding

We have reported (Ingle, Nezamis and Morley, 1951*b*) that the stimulation of muscle greatly accelerates the disappearance of intravenously administered glucose in the severely diabetic rat. Preliminary studies of the effect of muscle work upon the metabolism of glucose in the presence and absence of insulin are under way.

Summary

A technique for the study of work performance in the rat has been described. The gastrocnemius muscle is weighted with 100 grams and is made to contract five times per second by faradic stimulation. A normal anæsthetized rat can sustain a high rate of work for several days. The ability to work is lost within a few hours following adrenalectomy. Work performance can be sustained by the administration of adrenal cortex extracts and a little less well by the 17,11-oxygenated steroids of the adrenal cortex. This procedure is used as a research tool in studies on adrenal cortex physiology and in studies on other deficiency states.

REFERENCES

ADDISON, T. (1855). *On the Constitutional and Local Effects of the Disease of the Suprarenal Capsules* London.

ALBANESE, B. (1892). *Arch. ital Biol.*, 17, 239.

1947) demonstrated that the adrenal steroids can be ranked in the following descending order of activity when assayed in the muscle-work test: hydrocortisone, cortisone, corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone. The data of our current studies are in agreement. This order of relative potency is identical with that obtained

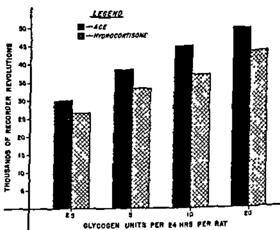


FIG. 4 A comparison of the total amounts of work done by adrenalectomized-nephrectomized rats given equivalent doses (glycogenic activity) of adrenal cortex extract (ACE) or hydrocortisone by continuous intravenous injection. Averages of 15 rats per group.

in the liver glycogen deposition test (Pabst, Sheppard and Kuizenga, 1947).

Other Possible Applications

Hypothetically, the work test should be useful in testing replacement therapy for any deficiency state which is characterized by muscular asthenia. The rat fatigues within a few hours following hypophysectomy. The administration of corticotrophin almost completely restores the capacity of the hypophysectomized rat to work (Ingle, Li and Evans, 1944). The work-test may provide a useful index of the effect of various fractions of corticotrophin upon the secretory

[illegible]

lactation completely in their rats after adrenalectomy with Compound

[illegible]

sources there was some contamination with hydrocortisone This was not recognized until cortisone was synthesized In my own earlier work with cortisone isolated from the gland we found biological responses with certain doses, but with the synthetic product we had to give nearly twice as much to get the same effect. I think part of the difference was due to use of the non-esterified compound as compared with the acetate, but it is also possible that the amount of hydrocortisone present with the cortisone had an important effect

BUSH. It seems possible from recent work at the Courtauld Institute that an even more important contamination of cortisone isolated from

[illegible]

those studies we treated the animals for one or for two weeks after adrenalectomy before we subjected them to the work test. The ones receiving the mixture worked better than those on cortisone alone, but the level of work output remained subnormal

INGLE, D. J., LI, C. H., and EVANS, H. M. (1944) *Endocrinology*, 35, 91.

INGLE, D. J, and NEZAMIS, J E. (1949) *Amer. J. Physiol.*, 156, 365

INGLE, D. J., NEZAMIS, J. E., and MORLEY, E. H. (1951*a*). *Proc. Soc. exp. Biol Med.*, 78, 79.

INGLE, D J., NEZAMIS, J. E., and MORLEY, E. H. (1951*b*). *Amer. J. Physiol*, 165, 469.

MASON, H. L., MYERS, C S., and KENDALL, E. C. (1936). *J. biol. Chem*, 116, 267.

PABST, M. L, SHEPPARD, R, and KUIZENGA, M. H. (1947). *Endocrinology*, 41, 55.

STEIGER, M., and REICHSTEIN, T. (1937). *Helv chim. Acta*, 20, 1164.

DISCUSSION

H J. ROBINSON: Are your adrenalectomized rats maintained on saline when they go downhill so rapidly?

INGLE. They were put to work immediately after adrenalectomy so that there was no maintenance period The untreated adrenalectomized control animals which I showed received saline by continuous intravenous injection during the time they were working.

COPE: In so far as you get better effects from the crude adrenal cortical extract than from either E or F, the assumption is that there is something there producing a better effect Have you tried whether adrenal cortical extracts potentiate the action of E and F, possibly in small amounts?

[illegible] Dr.

[illegible] ly we

[illegible] work

test.

GADDUM Is the evidence really sound that the amorphous fraction is very active? I have heard some doubts cast upon it. Have you any doubts at all?

[illegible] I have known

at least according to the majority of reports hitherto published. It has been suggested that compound S (the 17-hydroxy derivative of DOC) *might fulfil the rôle of the natural* salt hormone, but its biological activity appears to be disappointingly low (Masson, Corcoran and Page, 1950). The fact that cortisone, when given in sufficient doses, will affect sodium metabolism in exactly the same way as DOC (Roberts and Pitts, 1952) may argue against the dual nature of cortical secretion. On the other hand it is certain, and has just recently been re-emphasized in experiments by Ingle, Nezamis and Morley (1952), that Compound F is inferior to whole adrenal extract in restoring functional deficiency produced by adrenalectomy. Many workers have pinned their hope on the "amorphous *fraction" to account for this discrepancy in* efficacy, but conclusive evidence to show that this fraction contains potent unknown substances normally secreted by the gland is still lacking. The very interesting experiments of Tait, Simpson and Grundy (1952) on fractionated adrenal extracts demonstrate the presence, in such extracts, of an unknown compound with high activity on mineral metabolism. There is no evidence as to whether the natural adrenal secretion contains this new substance (or something similar to it). Spencer (1950) reported salt retaining properties of serum from adrenal blood of a dog. The activity in terms of DOC was very high, equivalent to 4 μg./ml., but unfortunately the author did not follow the matter up, nor did he state the potency of the same material in terms of an 11-hydroxylated compound. It ought to be possible to obtain evidence on the nature of the products of glandular secretion by the comparison of the same sample of adrenal effluent in a test *specific for steroids with O on C-11 and in a test which* will also or predominantly respond to DOC. Unfortunately, so far, assays using effects on electrolyte metabolism have proved very difficult to standardize and to reproduce. Attempts with Spencer's method on the sodium retention of adrenalectomized mice are in progress in Edinburgh, but the technical difficulties have not yet been mastered. The

OBSERVATIONS ON COMPARATIVE BIOLOGICAL ASSAYS OF ADRENAL CORTICAL STEROIDS

MARTHE VOGT

THE most successful field for comparative biological assays of adrenal corticoids has hitherto been the comparison of pure corticoids either with each other or with cortical extracts. You are all familiar with the data represented in Table I, according to which deoxycorticosterone and the steroids oxygenated in the C-11 position are contrasted by means

Table I

Tests	*DOC*	*Cpds. with O on C-11*
Survival	+++	+
Blood urea	+++	+
Na Retention	+++	+
Ingle's muscle work test	+	+++
Glycogen deposition	+	+++
Fall in lymphocytes or eosinophils	0	+++

of two groups of biological tests. In adrenalectomized animals DOC is the more active compound in tests using survival, urea retention, and sodium retention; whereas it is inactive or very nearly so when the tests used are the muscle work performance (with Ingle's method), the glycogen deposition or a fall in circulating lymphocytes or eosinophils.

One of the problems which exercise the mind of many workers at present is whether the natural secretion from the gland contains compounds which fall into these two groups. The belief, held for a long time, that DOC is the natural hormone regulating the mineral metabolism, became increasingly unlikely when chromatographic analysis of blood draining the adrenal glands did not reveal this compound,

and cortical extracts in most of the cold tests. One ml. of this extract was equivalent to 30 μg. Compound E in the mouse eosinophil test.

The comparisons were made with two objects in view first, to get figures for the secretion rate in terms of Compound E; and secondly, to try and see whether changes in the rate

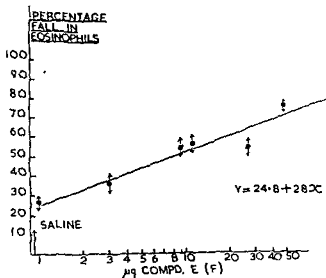


FIG 1 Dose/response curve for Compounds E and F in the mouse eosinophil test

of secretion would be reflected in the same way in both tests. Fig. 1 shows the dose/response curve for the assay of Compound E in the mouse eosinophil test. Identical results, incidentally, are obtained with Compound F. The figure immediately shows the difficulty inherent in this work: the dose/response curve is so flat that, in order to obtain significant differences between responses, the dose has to be increased by a factor of about 4 when 10 mice are used per group Since it is impracticable to increase the number of animals, it is obvious that changes in the rate of secretion

comparative assays on which I can report today were carried out by Dr. Bibile and myself and employ the fall in mouse eosinophils* as a test for substances with an O at C-11, and the *survival test at low temperature* as a test in which the animals respond more indiscriminately to adrenal corticoids. There are two reasons for suspecting that survival at low temperature is not prolonged exclusively by compounds carrying an oxygen in the C-11 position: one is the lack of parallelism of dose-response curves obtained with adrenal extracts and with Compound E, the other is the efficacy of DOCA in the "cold test." This latter argument has, however, to be used with caution. Though the survival time of such rats can be prolonged by DOCA in doses which are only two to three times larger than the amounts needed of Compound E (about 20 μg. DOCA as compared with 8 μg. Compound E), the DOCA has to be given on the day preceding the exposure in order to be effective. Such a delayed action may be due to the slow absorption of DOCA. That this explanation may, however, be incorrect is suggested by the inability of the water-soluble DOC glycoside to protect unless the dose, given on the day of exposure, is about 60 times that needed of Compound E. Even allowing for the greater molecular weight of DOC glycoside, this is a large discrepancy. One possible explanation might be that the derivatives of DOC may not act on their own but only after oxidation in the body.

In spite of some uncertainty, then, about what action of corticoids is measured in the "cold test," a number of samples of adrenal plasma obtained from perfused adrenals or from glands *in situ* were extracted with ethyl acetate, the extracts purified, the steroids transferred to water and an estimation made of their potency in depressing the number of eosinophils in mice and in prolonging the survival of rats kept in the cold. Compound E was used as standard in the mouse test,

*The adrenalectomized mice were maintained with DOCA implants and we are greatly indebted to Messrs. Organon for a generous supply of 5 mg DOCA pellets.

of KCl, on the assumption that the chances of stimulating the secretion of a hormone acting on mineral metabolism—if there is such a hormone—might be greater with KCl than with ACTH. It is in these comparisons that the limitations of the assays made themselves most felt. The increases produced by KCl were often hardly large enough to be demonstrated when the assays were done by the cold test, so that the chances of detecting them in a test with even poorer discrimination between doses were slender indeed. There were two experiments in which small increases in hormone yield shown by the "cold test" were reflected in similar, but non-significant increases when the assay was done by the eosinophil test, so that the impression was created that the activity measured was always due to secretion of compounds with an oxygen at C-11. Whether this be due to the limitations of the methods or to the fact that adrenal secretion really consists predominantly of substances akin to compounds E and F, these assays have so far not produced any evidence for the dual nature of cortical secretion.

REFERENCES

INGLE, D J., NEZAMIS, J E., and MORLEY, E. H (1952) *Endocrinology*, 50, 1.

MASSON, G M C, CORCORAN, A. C, and PAGE, I H. (1950). *Endocrinology*, 46, 441.

ROBERTS, K. E, and PITTS, R. F (1952) *Endocrinology*, 50, 51

SPENCER, A. G. (1950). *Nature*, 166, 32

TAIT, J. F., SIMPSON, S A, and GRUNDY, H M (1952). *Lancet*, 1, 262, 122.

DISCUSSION

SEGALOFF: I'm a little troubled about drawing conclusions about

miscible ones like propylene glycol or urea-urethane solution, with deoxycorticosterone itself?

VOGT: I haven't tried those because they fall out of solution immediately when you inject them, and give just a suspension. I perfectly agree that the metabolism of the glycoside may be completely different

would have to be very large in order to be reflected in significant differences in the fall of the eosinophils. The threshold is about 1 μg. of Compounds E or F, whereas 40 μg. of DOC glycoside is without effect.

Table II shows the results of a series of such comparative estimations of adrenocortical secretion. In the perfused

Table II

CORTICAL SECRETION IN THE DOG (μg Cpd. E/g./min)

PERFUSED ADRENAL				ADRENAL IN SITU		
No. of Exp.	No. of sample	ASSAY ON Mice	Rats	No. of Exp.	Mouse Assay	REMARKS
3	1	6.9	>3.3	0	9.7	Splanchnics Intact
	2	1.9	>3.3			
4	1	1.9	>3.1	1	7	Splanchnics Cut
	2	2.2	>3.1			
	3	2.2	>3.1			
5	1	5.5				
	2	6.0				
	3	9.1	11.3			
6	1	4.5	3.9			
	2	2.1				
7	1	2.1				
8	1	1.5				
9	1	3.0	5.1			
	MEAN	3.7	>4.5			

gland, the figures vary little; the mean obtained in terms of Compound E per g. per minute appears a little smaller by the mouse assay (fall in eosinophils) than by the rat assay ("cold test"), but the difference is non-significant. As expected, the gland *in situ* yields a greater amount of hormone than the isolated gland, especially when the splanchnic nerves are left intact.

In some of these experiments, an attempt was made to increase cortical secretion in the perfused gland by means

ASSAY OF GLUCO-CORTICOIDS

J. A NISSIM

THERE are now a number of methods for the bioassay of gluco-corticoids, the most important of which are the rat glycogen method, the mouse glycogen methods of Venning, Kazmin and Bell (1946) and of Dorfman, Ross and Shipley (1946), and the method measuring the reduction in blood eosinophils (Speirs and Meyer, 1951). There is no doubt that methods employing the mouse are far more sensitive than those employing the rat. The mouse eosinophils method seems to be superior both in sensitivity and in precision, and though the author has no personal experience of this method, the remarks which will be made on the assay of gluco-corticoids are pertinent to this method as well. The author's experience in the bioassay of gluco-corticoids was gained whilst investigating the conversion of steroids by adrenal tissue. Two main points of interest have emerged from this study of bioassay technique: one with regard to the anæsthesia used in the operation of adrenalectomy; the other concerning the assessment of the accuracy and merit of different bioassay procedures.

Anæsthesia

The first 250 mice were adrenalectomized under combined pentobarbitone and ether, but the results left room for improvement. After that, a combination of local and intraperitoneal xylocaine was used, the intraperitoneal dose producing anæsthesia of the peritoneum as well as a state of stupor during which the operation could be carried out. The animals suffered less discomfort and shock than with ether, and this is supported by the mortality figures up to the fourth

from that of either *free* deoxycorticosterone or the acetate But all I wanted to say is that it does make you a little suspicious.

SEGALOFF. How did you decide on the end-point for the cold test? That was the major difficulty we encountered in trying to use that test

VOGT You mean, recognizing when they were dead? In most animals there is no doubt, but in those where there is doubt, we simply take an arbitrary end-point· when the corneal reflex disappears, we call the rat dead

PERRY I noticed you didn't claim any difference between 3 7 and >4 5. It certainly seems rather significant that 6 out of the 8 values for the rats were greater than those for the mice.

VOGT: Yes, but I wanted to be on the safe side. I didn't put the fiducial limits of those figures in the table, and I think if you had seen them you wouldn't like to draw any conclusions.

number of animals required in an assay to reach a particular level of accuracy.

In the present experiments on the bioassay of gluco-corticoid material, the mouse glycogen method of Venning was used. Briefly, this method involves the fasting of adrenalectomized mice for 22½ hours, during the last 6½ hours of which the material to be assayed is injected in divided doses. The animals are then killed and their liver glycogen determined. Venning *et al.* used a pure strain of mice from their "own colony". In the absence of breeding facilities, the present investigation had to be carried out with male white mice obtained from reputable dealers, but the purity of the strain could not be relied on. Results showed marked variation in the liver glycogen levels of adrenalectomized control mice as well as of those injected with gluco-corticoids.

The mean liver glycogen of 15 groups of 10 control adrenalectomized mice ranged from 2·026 to 29·199 mg./100 g body weight, with a mean of 12·4 ± 1·8. The coefficient of variation was 178·0 per cent, and values of liver glycogen ranged from 0·3 to 138·3 mg. Forbes, Griswold and Albright (1950) also recorded unduly large numbers of "positive controls", i.e. individual values exceeding 9 mg./100 g. in control groups, and found that these high values were eliminated by substituting a bran-containing diet in the last 15 hours before the fast. In the present experiments, the results of Forbes *et al.* could not be confirmed. Sixty-one adrenalectomized control mice put on the Forbes régime gave a mean of 12·48 ± 1·53 mg. and a coefficient of variation of 181·25 per cent (Figs. 1 and 2).

In biological assays, coefficients of variation greater than about 25 per cent are very troublesome and usually denote a multiplicity of variables. An attempt was made to determine the reason for this high variation. Accessory adrenal rests and faulty adrenalectomy were excluded by careful post-mortem examination. Liver glycogen levels were then studied in normal non-adrenalectomized mice, in fasted non-adrenalectomized mice, and in fasted non-adrenalectomized mice injected with Eucortone. All three groups gave a

post-operative day. For the 250 mice anæsthetized w ether the mortality was 20 per cent, whilst for 1737 m adrenalectomized with xylocaine it was only 6·8 per cent

Mice weighing 20–26 g. received 0·15 ml. of a 1 per c xylocaine solution (without adrenaline) subcutaneously a 0·15 ml. intraperitoneally. With a small shift in the directi of the needle, half the subcutaneous dose was placed over t right lumbar peritoneal incision, and half over the le Adrenalectomy was carried out 5–10 minutes after t injection of xylocaine. The tail of the mouse was strapp with adhesive plaster on a cork board, whilst an assista held the animal by the ear.

Procaine was found unsuitable when used in the same w as xylocaine, as the stupor was then associated with consid able excitement

Selection of Bioassay Method

With the different methods available for the bioassay gluco-corticoid activity, the problem of selecting a suital one for a particular investigation is not very easy. If se sitivity of assay is the primary need, the mouse glycog methods are far superior to the rat method, but the latt has been said to give more accurate results (Pabst, Sheppa and Kuizenga, 1947; Dorfman, 1949). In reviews on bioass procedures, Dorfman (1949, 1950) compared the accuracy his mouse glycogen method with that of Venning *et al.* (194 using the index of precision, or, what amounts to the san thing, the error range of potency ratio. He arrived at th conclusion that Venning's method was the more accurat though the difference was not very great. The index o precision is equal to the square root of the mean square o error (or error of random sampling in an analysis of variance divided by the slope, i.e. s/b, and has been named λ b Gaddum (1933) The quantity $1/\lambda^2$ is proportional to th amount of information per test object. The figure obtaine for λ is therefore an excellent guide to the precision of th experiments, and the smaller its value, the smaller is th

Table I

Coefficient of Variation of Liver Glycogen Levels in Normal Non-fasted Mice, Normal Fasted Mice, and Normal Fasted Mice Injected with 0·1 ml. Eucortone

Group	*Number of animals*	*Period of fast in hours*	*Liver glycogen per 100 g mouse Mean±S E*	*Coefficient of variation*	*Remarks*
a	30	0	81·08±11 53	77 9	
b	30	24	16 80±3 70	120 5	
c	10	24	46 83±17 27	116 5	injected with Eucortone

The error contributed by the chemical estimation of glycogen was determined by control experiments and found to be negligible.

Attempts were then made to eliminate "positive controls" by a number of procedures, even at the expense of some loss of sensitivity. Forced exercise for a period of four hours prior to the assay and exposure to cold for a similar period failed to produce the desired result. The possibility that some gluco-corticoid may be evolved by the testis was investigated. Combined orchidectomy and adrenalectomy gave no better results. Pre-treatment with thyroxine was the only effective measure (mean 3·33±0 81), though the coefficient of variation remained high (132·5 per cent), and the mice were rendered totally insensitive to gluco-corticoid substances.

Mice obtained from a second dealer failed to give smaller variation.

In the assay of cortisone acetate a good regression line was obtained (Fig. 3), but at the expense of using 40 animals per group.

Lastly, liver glycogen levels in white Wistar rats showed low coefficients of variation which are not different from those of other workers (15–25 per cent). The coefficients of variation of mice used in the assay of cortisone on the same days, as control for the rats, averaged 60 per cent. Perhaps the most striking contrast was between the coefficient of variation of

similarly wide variation (Table I), which could not in this case be attributed to faulty operative technique. A group of non-adrenalectomized mice with a mean liver glycogen (10·94 mg. /100 g.) comparable to that of the adrenalectomized control group showed a variation coefficient of 197·5.

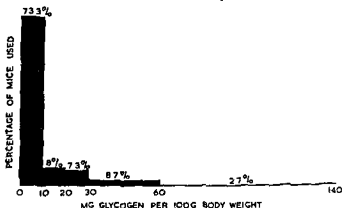


FIG. 1. Block diagram showing percentage distribution of liver glycogen levels in 150 fasted adrenalectomized control mice kept on ordinary stock diet.

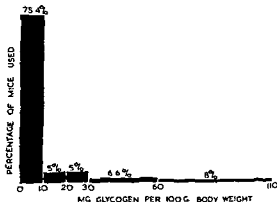


FIG. 2. Block diagram showing percentage distribution of liver glycogen levels in 61 fasted adrenalectomized control mice kept on "diet A+B" of Forbes *et al* (1950)

two workers must be taken into account when the merits of their methods are compared. Under such circumstances, the independent study of the square root of the mean square of error and of the slope yields more information than a comparison of the indices of precision. This is well illustrated in the comparison between the methods of Venning and of Dorfman. The smaller index of precision of the method of Venning *et al.* is seen to be due merely to a smaller variation, the slope being greater in the case of Dorfman *et al.* Had the coefficients of variation been reversed in the two methods, the indices of precision would have shown the ratio of 6·2 instead of being nearly equal Thus, it may be said that λ is an index of accuracy or precision of experiments, not of methods.

Table II

COMPARISON OF VALUES OF COEFFICIENT OF VARIATION, SLOPE, AND INDEX OF PRECISION FOR CORTISONE IN BIOASSAYS CARRIED OUT BY DIFFERENT WORKERS

	Name of author		
	Nissim	*Dorfman* et al (1946)	*Venning* et al (1946)
Average c.v for cortisone	106 825	56 80	20 825
Slope for cortisone (*b*)	12 9	22 23	9 59
Index of precision (λ) or *s*/*b*	0 505	0 158	0 137
√Mean square of error (*s*)	6 523	3 512	1 314
s/c.v ratio	0 06105	0 0618	0 0631

c v.=coefficient of variation

In conclusion, it is pointed out that if pure strains of mice are available, methods employing the mouse can give just as accurate assays as the rat method, if not more accurate. This may well be seen from the work of Venning *et al.* (1946) and of Speirs and Meyer (1951).

REFERENCES

DORFMAN, R. I (1949) *Ann New York Acad. Sci.*, **50**, 556.
DORFMAN, R I. (1950). In *Hormone Assay*, ed. by C. W. Emmens. New York. Academic Press.

liver glycogen in normal rats (6·5 per cent) and that in normal mice (77·9 per cent).

A comparative study was made of the coefficients of variation obtained in the bioassay of cortisone with those of other workers. The relative magnitude of this coefficient is characteristic throughout the experiments of each investigator. Since, as shown above, this variation is independent of the technique of adrenalectomy, and the error of the chemical

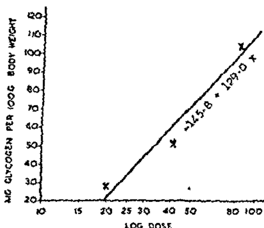


Fig 8. Logarithm dose-response regression line for cortisone acetate—from 3 points, 20, 40 and 80 micrograms.

estimation adds little to it, the average coefficient of variation is representative of the purity of the strains used by each investigator.

Table II shows a comparison of the coefficients of variation, the slope, and the indices of precision obtained for cortisone in bioassays by Venning *et al.*, Dorfman *et al.*, and the present investigator. The square root of the mean square of error (s) is shown to be proportional to the average coefficient of variation, so that the magnitude of the mean square of error in turn must depend practically solely on the strain of mice used. Differences in the coefficients of variation obtained by

geneticist, and has actually a true strain in the eugenic sense. Is that true?

Segaloff: Yes. Speirs is a geneticist. They had some 25 or 30 strains available at Bar Harbor and they selected the strain that worked best. Incidentally, I was quite amazed when I was told that it's easier here to get pure strain rats than mice, because you can buy pure-strain mice from almost any dealer in the States and pure strain rats are extremely difficult to get. We can actually purchase the most sensitive strain of mice from Bar Harbor. But I think we must remember that in experiments on carcinogenic hydrocarbons, the British workers are on record as saying they get less variation from market mice than with inbred strains. [illegible]
superior. [illegible]
neous an[illegible]
some thi[illegible]

Vogt: If the purity of the strain were very important in the mouse eosinophil test you would imagine that you would have smaller variations within litters than between litters, and that is not so

Segaloff: I think it would depend on the relative purity of the mice you start with. The possibility with the genetic differences that exist are tremendous. I am told that in mice it takes some 22 generations of brother to sister mating to get a homozygous stock

Vogt: I don't mean that the litters are homogeneous, but surely they ought to be more homogeneous than non-litter-mates

Segaloff: They *ought* to be.

Astwood: It seems to me that when divergent results between laboratories have been blamed upon strain differences in the past it has eventually proved to be not the case, and I wonder if it's not because [illegible]

[illegible]

between glycogen and total sugar.

Nissim. They arrived at the conclusion that the total sugar method was 5 times as sensitive as the liver glycogen. The coefficient of variation was 60 per cent of that of the latter.

Perry. Am I right in assuming that this distribution of liver glycogen values is extremely skew? It seems to me that the strain of mouse used may be important in this respect, since with the strain you are using a few animals appear to give responses very high in the scale. It also seems to me that the coefficient of variation is not a very good measurement of the scatter in these skewed distributions

Nissim The means of those groups were taken as well (i e. the mean

DORFMAN, R. I., ROSS, E., and SHIPLEY, R. A. (1946). *Endocrinology*, 38, 178.

FORBES, A. P., GRISWOLD, G. C., and ALBRIGHT, F. (1950). *J. clin. Endocrinol.*, 10, 230.

GADDUM, J. H. (1933). *Med. Research Council Special Report Series*, No. 183.

PABST, M. L., SHEPPARD, R., and KUIZENGA, M. H. (1947). *Endocrinology*, 41, 55.

SPEIRS, R. S., and MEYER, R. K. (1951). *Endocrinology*, 48, 316.

VENNING, E. H., KAZMIN, V. E., and BELL, J. C. (1946). *Endocrinology*, 38, 79.

DISCUSSION

SEGALOFF: Unless I am misinterpreting which one of the Venning tests you're talking about, the Dorfman and Venning tests are actually different, not only in animals, but in method: Dorfman uses an oil solution, Venning uses a solution in alcohol, and adds glucose to the injection medium. Dr. Venning does not employ pure strain animals, but a semi-inbred colony. Actually they found that temperature made more difference than the animals. We ourselves have had a great deal of difficulty with animals that come from the outside, and have been employing of late only F_1 animals bred in our own colony. I'm troubled that you're ascribing these differences to strains of animals when there are much greater differences in the actual assay procedures employed. With the Venning method, in our experience and I think the experience of others, from season to season, even using inbred strains of animals, you have to change the amount of glucose that you add to keep your percentage of positive controls within allowable limits. I wonder if that isn't possibly a greater contributing factor than the strain of animal.

NISSIM: My impression was that where you had high variability in the strain the method contributed comparatively little to the coefficient of variation. In particular, there is this high variation in the liver glycogen of normal mice, whereas in rats the coefficient of variation is very low. The contrast is very striking there, and shows that the error contributed by the method is very small. That was the object of doing normal animals.

SEGALOFF: Were the rats from your laboratory?

NISSIM: Yes.

VOGT: But would this not also show that mice are simply more sensitive to environmental conditions? Mice are after all preferred to rats for this work because they are more sensitive, but any animal which is more sensitive to the injection of corticoids would also be more sensitive to varying environmental conditions and therefore have inherent in it a greater coefficient of variation, whether the strain is pure or not.

NISSIM: That's true. But if you have a sensitive method the need for a pure strain is greater because any variation will show much more.

COPE: One query relevant to this that I might throw out to our American friends: I believe that either Speirs or Meyer is himself a

THE PAPER CHROMATOGRAPHY OF STEROIDS AND ITS APPLICATION TO ASSAY PROBLEMS

I. E. BUSH

THE word assay has a rather wide range of meaning and the method presented in this paper does not constitute an assay *in the sense* of a strict quantitative estimation of particular hormones. Instead it is a method of general application to the semi-quantitative estimation of a variety of steroids in blood, urine, and tissues. It is useful for exploratory experiments, and for routine use with some problems. When used for steroids containing the $\alpha\beta$-unsaturated 3-ketone group the method is capable of detecting 0 5 μg.; and over the range 0·5–5 μg. the semi-quantitative method of estimation used is in fact probably as good as, or better than, any bioassay capable of dealing with such small amounts. At present it is probably unsuitable for clinical work unless good facilities for chromatographic work are available.

Methods

Extraction of Blood

The blood sample (5–30 ml) is diluted with an equal volume of water and the hæmolysed blood extracted 1×6 volumes of ethyl acetate for five minutes The ethyl acetate extract is washed $1 \times \frac{1}{10}$ volume 0 2N-Na_2CO_3 and $1 \times \frac{1}{10}$ volume water. A drop of glacial acetic acid and 5 ml. absolute ethanol are added and the extract distilled to near dryness at 45°C. *in vacuo*. An air stream can be used during distillation. The distillation flask is well rinsed with 6 ml. light petroleum (b.p. 40–60°C.) and the petrol solution transferred to a 15 ml. glass-stoppered test-tube. The flask is washed again with 4–5 ml. petrol and then with 2 ml. 70 per cent ethanol, both solutions being transferred to the test-tube. All transfers

of ten groups instead of individual values) for the calculation of the coefficient of variation and the results were still high.*

CLARKE I should like to say that I should be hesitant to attribute differences between coefficients of variation wholly to differences in purity of strain Moreover, in the comparisons we gave yesterday breeds were compared within the same experiment, and each experiment was *done at the same time by the same people under the same conditions.* One of the values of using lambda as a basis for comparison is that one is usually interested in the precision of the estimate of relative potency, which can be expressed in terms of lambda, and differences in slope

is a

ceal

NISSIM: Yes.

[illegible] difficulty in our earlier experi-

quite an

*I should like to add that skewness does not necessarily argue against admixture of strains, if the distribution of the values in question tends to be *skewed in the normal population* (e.g., blood pressure frequency distribution in man).

known amounts of an $\alpha\beta$-unsaturated 3-ketosteroid. With clean extracts this method has an error of $\pm$15–30 per cent in the range of 1·5–15 μg. when the spots cover 1–3 cm.^{2} each.

For permanent records, the dried chromatograms can be photographed in ultraviolet light with a Wratten 2 B filter and an exposure of about five minutes. This filter is not selective enough for urine extracts but is excellent with blood extracts. The photographic method is more sensitive than the visual method, spots of $\frac{1}{4}$ μg. being easily seen on the photographs.

Application to Urine Extracts

The method can be applied to the examination of urine, though actual estimation is much more difficult due to the occurrence of large numbers of green, blue or pink fluorescent spots in some specimens.

In normal females one quarter of a 24-hour sample of urine is sufficient for the detection of a large number of reducing steroids and $\alpha\beta$-unsaturated 3-ketosteroids in different fractions. In pregnant females, patients with Cushing's disease, and some diabetics one-tenth of a 24-hour sample is sufficient.

Application to Other Tissues

Adrenal gland extracts can be satisfactorily separated and examined.

Recovery Values

Recovery of 11-dehydro-17-hydroxycorticosterone and 17-hydroxycorticosterone from blood ranged from 80–100 per cent with amounts of 5–30 μg.

Some Applications and Results

Adrenal Gland Extracts

These have been described previously (Bush, 1952). Apart from the recognized active cortical steroids, different commercial extracts contain from two to four other $\alpha\beta$-unsaturated

are made with pipettes. The flask is then rinsed thoroughly with 10 ml. ethyl acetate (warmed to 45°C.) and the ethyl acetate washing kept. The test-tube is stoppered and shaken with fairly vigorous inversions for five minutes. The petrol layer is drawn off by a pipette after settling of the layers. and discarded. Ten ml. dry chloroform and 1 ml. water are added to the tube and the tube shaken for five minutes. After settling, the upper emulsified layer is drawn off by pipette and the chloroform extract evaporated at 45°C. in a glass-stoppered tube with side-arm after the addition of a trace of Sudan red. The ethyl acetate flask washing is used to wash the extraction test-tube and then added to the main extract in the evaporating tube and concentrated at 45°C. to about 0·1 ml. This extract is taken up on a clean 5-strand lamp-wick and concentrated by the "wick" method (Bush, 1952) before deposition on Whatman No. 2 filter paper for chromatography.

Chromatography

Chromatography is carried out by the methods previously described (Bush, 1952), the best system for active cortical steroids being toluene-methanol:water, 10 7:3 (v/v) at 36°C.

Detection and Estimation

The chromatogram is dried and then sprayed with one of a variety of reagents. For active cortical steroids the reagent used is a solution of 0 5–1·0 mg. triphenyltetrazolium chloride in 100 ml. aqueous 2N-NaOH. After marking with pinholes the pink spots produced by reducing substances, the chromatogram is dried by heating in *still air* to 80°C. in an oven or by infrared lamps. When quite dry (with a slight yellowing of the edges of the paper) the chromatogram is examined in strong ultraviolet light using a *Wood's glass* filter. $\alpha\beta$-Unsaturated 3-ketosteroids give bright yellow fluorescent spots which are quite distinct from any other fluorescent substances on the chromatogram. Estimation is achieved by comparison of the fluorescence with spots of

secreting at a very high rate) responded by increasing its output of corticosterone much more than its output of 17-hydroxycorticosterone.

In cats, rats and sheep (Bush and Ferguson, unpublished) a much less polar substance very similar in properties to 11-hydroxyandrost-4-ene-3:17-dione has been found in adrenal venous blood. In cats appreciable amounts of 11-dehydrocorticosterone and 11-dehydro-17-hydroxycorticosterone have also been found.

Two of the $\alpha\beta$-unsaturated 3-ketosteroids more polar than 17-hydroxycorticosterone have been detected in the adrenal venous blood of some cats, dogs, and sheep (Bush and Ferguson, unpublished). Both give orange coloured dinitrophenylhydrazones and reduce triphenyltetrazolium chloride.

The rates of secretion observed in these experiments have all been very large and the absolute figures are so close to those of Vogt (1943) that it seems likely that the substances detected chemically will account for all the activity of adrenal venous blood in the Selye-Schenker cold test (Selye and Schenker, 1938).

In rabbits acutely hypophysectomized one hour before collecting adrenal venous blood the secretion rate was from one-fifth to one-tenth that observed in the (otherwise) intact animals. On giving intravenous ACTH the adrenal cortical secretion increased rapidly to the usual rates seen in animals subjected to the severe operation necessary for the collection of adrenal venous blood. The same behaviour was observed in isolated perfused adrenals from cats and ferrets when perfused with blood drawn from anæsthetized donors and circulated in the perfusion apparatus (Vogt, 1951) for 1–2 hours before the start of the perfusion. It seems likely that the adrenal cortex in most mammalian species can secrete at rates of at least 10 times the basal rate during severe stresses.

Only two cases of adrenal hyperfunction in man have been studied so far. Thanks to the courtesy and co-operation of Mr. L. R. Broster, adrenal venous blood has been collected

3-ketosteroids more polar than 17-hydroxycorticosterone. In addition, two other reducing steroids more polar than 17-hydroxycorticosterone but giving no fluorescence with NaOH are present in considerable amounts.

Adrenal Venous Blood

Adrenal venous blood has been collected from a variety of mammalian species *in vivo* with the splanchnic nerves usually intact. The results of these experiments were fairly clear-cut in showing that under the stress of the operation necessary for this type of experiment (similar to that of Vogt, 1943) all the species examined secreted relatively enormous amounts of 17-hydroxycorticosterone, corticosterone, or a mixture of the two. Some species secreted only corticosterone, the monkey secreted only 17-hydroxycorticosterone, but most species secreted a mixture of the two compounds (Table I).

Table I

Ratio of Major Steroids in the Adrenal Effluent Blood of Various Species

	Ratio $\frac{\textit{Compound F}}{\textit{Compound B}}$	*Maximum secretion rates mg /kg /24 hr / animal*
Monkey	>20	3 5–5 5
Dog	6	3 0–6 0
Cat	4–6	3 5–8 5
Sheep	15–20	
Ox	1	(2–3 5)
Ferret	1 5	1 5–4 5
Rabbit	<0 05	1 5–3 5
Rat	<0 05	8–24

The ratio of these two compounds in the adrenal effluent blood appeared to be a characteristic of each species studied and was never observed to change appreciably during considerable changes in total secretion rate. This behaviour of the adrenal cortex, however, is probably limited, since in a few experiments in which very large doses of ACTH were administered in a very short time to cats, the adrenal (already

much less reliable in this field due to the very variable purity of different urine extracts, and the complexity of the chromatograms obtained with urine extracts is considerably greater than with those of adrenal venous blood. However, certain regular and dominant features are already apparent.

Table III

STEROIDS FOUND IN URINE αβ-UNSATURATED KETONES AND REDUCING SUBSTANCES

	Immediate extraction at pH 1	*Glucuronidase hydrolysis (spleen)*
Principal αβ-unsaturated ketones/24 hr.	Compound L 12–50 μg Compound F 8–50 μg Amorphous A I 4–80 μg Amorphous A II 2–12 μg Amorphous A III 0–6 μg	Compound E 4–12 μg Compound F 2–40 μg (minimal amounts of A I, II, III) Intermediate E/F 4–100 μg
Principal reducing substances	The above compounds	The above compounds ? *allo*Pregnan-3 11 17-triol-11 20-dione Unidentified
Small quantities of other compounds	Five other αβ-unsaturated ketones	Three other reducing substances

Eighteen 24-hour urine samples from different women have been analysed in the following way. Equal fractions have been: (*a*) extracted immediately after acidification to *p*H 1 with HCl, (*b*) extracted after 24 hours at *p*H 1; (*c*) extracted after treatment with calf spleen glucuronidase. All extracts were made with chloroform and the extracts washed with $0{\cdot}2$ N-Na_2CO_3 and water before chromatography.

Examination by paper chromatography in the system benzene: methanol: water, 10.5.5 (v/v) at 37°C. showed that four prominent reducing substances were obtained in the glucuronidase-treated fractions which did not give a fluorescence with NaOH. These were present in all eighteen

from the left adrenal vein during operations for adrenalectomy and analysed by paper chromatography of the ethyl acetate extracts of this blood (Table II). In one case, a woman suffering from Cushing's disease with hirsutism and some degree of virilism, the adrenal blood contained 17-hydroxycorticosterone and corticosterone in a ratio of about 20 : 1 and about one part of a substance very similar to 11-hydroxyandrost-4-ene-3:17-dione. In the other case, a woman suffering from long-standing severe virilism, with facial hirsutism, hypertrophied clitoris, and no visible breasts, the

Table II

Human Adrenal Venous Blood

Case	*Condition*	*Steroids and proportions found*
J.D (♀)	Virilism for 20 years. Breast atrophy, no obesity, hirsutism, amenorrhœa, enormous clitoris Malignant adrenal	Compound F 1 Unidentified 1 1 Unidentified 2 3
B.M. (♀)	Cushing's disease with hirsutism	Compound F
R.F. (♀)	Cushing's disease and virilism (Broster's type II)	Compound F 20 Compound B 1 Unidentified 2 1

picture was quite different. The adrenal venous blood from this patient contained much more of the substance similar to 11-hydroxyandrost-4-ene-3.17-dione than of 17-hydroxycorticosterone, and a small amount of an unidentified steroid previously only found in urine.

Steroids in Urine

Although there have been only a small number of complete experiments carried out so far on urine, the results obtained on urine from normal and pregnant women already suggest some interesting differences in the steroids found in different *types* of urine extract (Bush, de Courcy and Gray, 1952; and unpublished observations). Quantitative estimations are

Table IV

17-HYDROXYCORTICOSTERONE IN PERIPHERAL BLOOD

Case		Condition	Blood sample (arm vein)	Concentration in µg /100 ml blood
M.	(♀)	Pregnant 8 months	30 ml	13 4
M K.	(♀)	39 hr post-partum toxæmic pregnancy	30 ml.	1 7
J	(♀)	37 weeks toxæmic pregnancy	30 ml	23
V.B.	(♀)	Cushing's syndrome, very diabetic	25 ml	24
S B	(♀)	*Metastases after removal of adrenal tumour 1 year*	50 ml	16
L	(♀)	Re-admission after removal of adrenal tumour 9 mths	30 ml	20
F B	(♂)	Cushing's disease	50 ml	20
B C.	(♀)	Cushing s syndrome	30 ml	11
C.	(♀)	Cushing s syndrome	35 ml	23

concentration of both 17-hydroxycorticosterone and corticosterone was observed before delivery (27 µg./100 ml and 83 µg /100 ml). Twenty hours after delivery corticosterone was not detectable in the peripheral blood (less than 3 µg /100 ml) and 17-hydroxycorticosterone was present in a concentration of 17 µg /100 ml.

Discussion

The results above are given mainly to show some of the applications of the method briefly outlined here and given in greater detail in another paper (Bush, 1952). The biological significance of the results on human urine and peripheral blood will only become clear when more cases have been studied. However, some idea of what to expect in the assay of steroids in blood and urine has been gained, and this is likely to be useful when considering the simplification of such assays that is necessary for clinical work. For instance, it seems likely that very different types of steroids are released by enzymic and acid hydrolysis of urine and that nine reducing steroids, five of which are also αβ-unsaturated

patients and were considerably *increased in the pregnant* women.

The extracts obtained by extraction immediately after acidification to pH 1 contained very small amounts of these four reducing substances but regularly contained four substances giving strong yellow fluorescence after NaOH treatment, as well as reducing triphenyltetrazolium chloride. Two of these seem to be identical with 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone and the other two seem to be identical with two of the compounds more polar than 17-hydroxycorticosterone found in adrenal gland extracts. These four compounds were also found in the glucuronidase-treated fractions in large amounts in cases of pregnancy or diabetic pregnancy, but in samples from normal women only 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone were present in appreciable amounts in these fractions. A fifth substance giving a strong yellow fluorescence after NaOH treatment, intermediate in polarity between 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone, occurred in large amounts in the glucuronidase fractions of all 18 patients.

At least five other presumed $\alpha\beta$-unsaturated 3-ketosteroids were detectable in some of the urines from pregnant women.

The extracts prepared by chloroform extraction after standing at pH 1 for 24 hours only contained small amounts of $\alpha\beta$-unsaturated 3-ketosteroids and no detectable reducing *substances in most cases.*

Human Peripheral Blood

The results obtained on a variety of substances are given in Table IV. Most of them were cases of Cushing's disease and some of them cases of normal or toxæmic pregnancy. No values have been obtained for normal humans as yet.

In all cases 17-hydroxycorticosterone was detectable in 25–30 ml. samples of blood, and concentrations as high as 23 μg./100 ml. blood were observed. In one case of toxæmia of pregnancy, not recorded in the table, a remarkably high

For instance, isomers differing only by small changes of orientation in the molecule are not readily separable by partition chromatography, and other possible confusions (Hanes and Isherwood, 1949, Campbell and Work, 1952) must be continuously guarded against by using as many cross-checks of identity and homogeneity as possible. Nevertheless, the methods outlined above have proved to be both versatile and useful in investigating various aspects of adrenal cortical secretion and can probably be successfully extended to other problems involving the assay of steroids in body fluids and tissues

REFERENCES

BUSH, I. E. (1952). *Biochem J.*, 50, 371.

BUSH, I E., DE COURCY, C, and GRAY, C. H. (1952) (In press)

BUTT, W. R., MORRIS, P., and MORRIS, C J. O R. (1949) *1st Int Congr Bioch. Abstr*, p 405

CAMPBELL, P. N., and WORK, T. S (1952) *Biochem. J*, 50, 449

HANES, C. S., and ISHERWOOD, F A (1949) *Nature, Lond*, 164, 1107.

NELSON, D. H., and SAMUELS, L T (1952). *J clin Endocrinol*, 12, 519

SELYE, H., and SCHENKER, V. (1938). *Proc Soc exp. Biol*, N.Y., 39, 518

TAIT, J F., SIMPSON, S A, and GRUNDY, H M (1952) *Lancet*, 262, 122.

VOGT, M. (1943) *J Physiol*, 102, 341.

VOGT, M. (1951) *J Physiol.*, 113, 129.

ZAFFARONI, A, BURTON, R B, and KEUTMANN, E. H (1950) *Science*, 111, 6

DISCUSSION

NELSON I'd like to describe briefly the method that we've used for 17-hydroxycorticosteroids We have changed our method quite a bit since we corresponded with Dr. Bush Very simply, it consists of ex-

these cases we have evidence for a compound which apparently is neither F nor B, and which we are now investigating. This was in a case which had an adrenal tumour at operation.

BUSH How does the polarity of this compound compare with F and B?

3-ketosteroids, make up the bulk of the reducing steroids in urine obtained by these methods of extraction.

The results on peripheral blood are in good agreement with the results of Nelson and Samuels (1952) using a different method of estimation. They also suggest by direct experiment that, at any rate in conditions of increased adrenal cortical activity, 17-hydroxycorticosterone is the principal steroid present in the circulating blood, and that the concentration of this substance in pregnant women can be as great as those found in cases of moderately severe Cushing's disease.

The general method of chromatography described here is based on the solvent system originally used by Butt, Morris and Morris (1949) on Kieselguhr columns for the estimation of progesterone. The use of an elevated operating temperature is not essential (Henly, personal communication) with most of the solvent systems used but greatly increases the speed and quality of the results obtained. By varying the proportions of the solvents used almost any type of steroid as polar or more polar than progesterone can be handled. Unlike Zaffaroni's method (Zaffaroni, Burton and Keutmann, 1950), no previous treatment of the paper is needed, definite R_F values can be obtained, and useful chromatograms obtained in a total of five hours equilibration and running.

The major disadvantage of this method at present is that steroids other than the αβ-unsaturated 3-ketones cannot be detected in amounts of less than 5 μg. by any colour reaction yet described. The possibility that steroids lacking this grouping are being overlooked when using the NaOH fluorescence test is unfortunate. It is very probable for instance that the "salt-active" substance found in adrenal extracts by Tait, Simpson and Grundy (1952) does not contain this grouping, and the search for this and other compounds must always involve biological as well as chemical assay until sufficient evidence has been obtained to enable simplification of the assay method.

As with other assay methods, the limitations of this and other chromatographic methods are serious in some respects.

BAYLISS Dr. Bush, when you were extracting urine, what did you get out at neutral pH as compared to the ones you acidified at pH 1 and extracted with chloroform?

BUSH: This work on the effect of hydrolysis on urine was done in conjunction with Professor Gray and Miss de Courcy Generally, when urine was extracted at neutral pH and then acidified and extracted immediately, and again after 24 hours, we found that almost all the $\alpha\beta$-unsaturated ketonic steroids were found in the neutral fraction. Exceptions to this were urines containing large amounts of steroids and urines from some pregnant women

BAYLISS: Your enzyme isn't inactivated by the organic solvent left in the urine?

DE COURCY We remove the chloroform by bubbling nitrogen through the urine.

NELSON It's between F and B.

BUSH A woman with virilism whom we studied also had that compound, in very small amounts.

NELSON: I would like to emphasize that we are somewhat hesitant to take a run on the paper chromatogram and accept similarity of polarity as absolute evidence that we have a particular compound I think we should all be very hesitant to say that a compound is F or B, just on this basis.

BUSH: Of course. At its worst a sceptical chemist will describe chromatography as smudges on blotting paper, and won't accept such results as sufficient for purposes of identification The Compound F found in dog adrenal venous blood, however, has been exhaustively checked by a variety of different methods. We have shown that the acetate flows at the same rate as that of Compound F and that the compound reduces triphenyltetrazolium chloride, you can do this last test simultaneously with the caustic soda test. We have also shown that the oxidation

carbazone of the compound in adrenal venous blood is also chromatographically identical with that of pure Compound F.

This complete check has been done only on the dog, but on every chromatogram the reducing substances are detected with triphenyltetrazolium, then the paper if dried off, and $\alpha\beta$-unsaturated 3-ketones detected by the NaOH fluorescence test. So we have in fact got good evidence in every case (1) that the polarity of the two substances is very similar; (2) that they're reducing substances, (3) that they're $\alpha\beta$-unsaturated ketones with at least two rings I think that it's unlikely that any other substances would interfere in that battery of tests

of the other compounds

COPE There is another possibility. One might be collecting other

A preliminary series of 20 specimens was examined by this method and results compared with those obtained by the colorimetric method of Wick *et al.* (1951). The colorimetric results were generally higher than the polarographic results but the correlation was good, except for two very high results

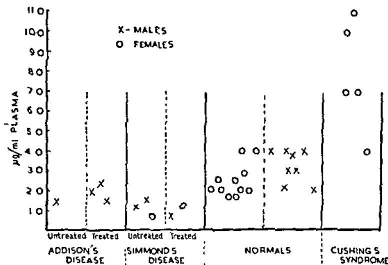


FIG. 1. *The plasma 3-ketosteroid level in normal individuals and in patients with Addison's disease, Simmonds' disease and Cushing's syndrome*

by the colorimetric method. When these were repeated they agreed with the polarographic results.

The values for 3-K.S. in normal subjects ranged from 1·7–4·0 µg./ml. plasma (Fig 1). Repeated determinations in five patients showed no significant diurnal variation. The values in a small series of untreated patients with Addison's and Simmonds' disease ranged from 0·7–1·5 µg./ml. plasma, while in treated patients they ranged from 0·7–3·0 µg./ml. In Cushing's syndrome values of 4 0–11·0 µg./ml. plasma were obtained.

DETERMINATION OF 3-KETOSTEROIDS AS A MEASURE OF ADRENAL CORTEX FUNCTION

W. R. BUTT and A. C. CROOKE

SEVERAL methods have been described for the determination of corticoids in blood. Many are tedious, lack sensitivity and specificity, and are unsuitable as routine clinical tests of adrenal function. Wick, Hillyard and Mackay (1951) described a method for the determination of reducing steroids in blood, [illegible]
has repor [illegible]
bioassay [illegible]
and Boyle, 1950) and the chemical determination of 17-hydroxycorticosteroids (Nelson, Samuels, Willardson and Tyler, 1951) appear to be better methods.

We have attempted to measure adrenal function by the polarographic determination of $\alpha\beta$-unsaturated 3-ketosteroids (3-K.S.) in blood. This group of steroids includes all the known physiologically active corticosteroids, and the only steroids of this group so far isolated from blood have been corticosteroids. Blood levels of the 3-K.S. progesterone and testosterone have previously been shown to be so low that they are not usually detectable by our method (Butt, Morris, Morris and Williams, 1951).

Method and Results

The method used has been described previously (Butt and Crooke, 1952). Plasma from 20 ml. whole blood was extracted by the method of Wick *et al.* (1951), followed by a micro-Girard separation of ketonic material. The polarographic determination was done on the Girard hydrazone, using deoxycorticosterone as a standard. The whole procedure can be conveniently completed in about 6 hours.

would seem to indicate that the latter are not the only circulating cortical steroids produced by ACTH.

We are now investigating the bound 3-K.S. in blood. We already have evidence that some free 3-K.S of adrenal origin may not be measured by our method. Many extraction

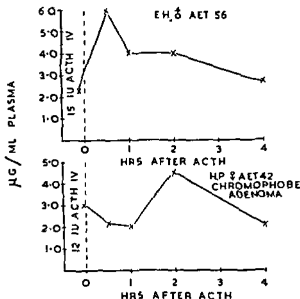


FIG 2 The plasma 3-ketosteroid level at varying intervals of time after the intravenous injection of ACTH into a normal individual and into a patient with Simmonds' disease

procedures, including our own, contain a petroleum ether washing and this is usually discarded. It contains a fraction, however, which appears to be important One patient who was given 25 i.u. ACTH intravenously showed no rise in 3-K.S. level, but when the petroleum ether soluble fraction was measured it was found that the 3-K.S. rose strikingly after 1 hour. They were maintained at a high level until 2 hours and returned to normal at 4 hours after injection.

One patient with Cushing's syndrome was studied during surgical treatment. Her plasma 3-K.S. levels were 11·0 μg./ml. and 10·0 μg./ml. on two occasions before operation. Twelve days after left partial adrenalectomy the level had fallen to 8·0 μg./ml. and three months after this it was 6·5 μg./ml. After right adrenalectomy the value fell to 1·0 μg./ml., when she developed symptoms of acute adrenal insufficiency due to premature withdrawal of cortisone. Ultimately, after a further course of treatment with cortisone the 3-K.S. rose to the normal range and reflected the improvement in her clinical condition. She is now well three months after the second operation and without added salt or cortisone. It is particularly interesting that before surgery this woman excreted normal amounts of 17-K.S. and corticoids, which shows that the plasma 3-K S. level was a much more accurate measure of cortical activity

The effect of ACTH on 3-K.S. levels was investigated in several patients. In one patient with normal adrenal function, 12 i.u. ACTH given intravenously produced a small rise after 30 minutes. In a second patient with normal adrenal function, a dose of 15 i.u. intravenously caused a significant rise of 2 3–6 0 μg./ml. after 30 minutes. The value remained at 4·0 μg./ml. until 2 hours and had fallen to 3·0 μg./ml. 4 hours after the injection. A dose of 12 i.u. given intravenously to a patient with Simmonds' disease (caused by a chromophobe adenoma of the pituitary) produced a significant rise (3·0–4 6 μg./ml.) 2 hours after the injection (Fig. 2).

Discussion

The values for normal people which have been obtained by our method are considerably higher than those of Nelson *et al.* (1951), measuring 17-hydroxycorticosteroids, but they are considerably lower than the glycogenic steroids estimated biologically in dogs by Paschkis *et al.* (1950). It is interesting, however, that ACTH causes as striking changes, relatively, in the 3-K.S. levels as in the 17-hydroxycorticosteroids, which

BUSH: Would saturated 3-ketosteroids give you that kind of polarogram?

BUTT: No, they would not.

C. J. O. R. MORRIS: There is one point I might add. We have tried Compounds F, E and S, and no 17-hydroxy-20-ketosteroid appears to give a 20-ketosteroid wave.

BUTT: Do you think that's due to steric hindrance?

C. J. O. R. MORRIS: I think it's rather more than that. I think they're structurally different substances. That is shown quite strikingly by infra-red absorption. The 17-hydroxy group also modifies the infra-red absorption of the 20-keto group.

ASTWOOD: Are these extracts pure enough so that you can determine an $\alpha\beta$-unsaturated 3-ketone by simple ultraviolet absorption?

240 mμ

BUSH: I think the trouble with ultraviolet absorption of crude ex-

instrument

NELSON: I might say that when we originally started our work on blood we were chiefly interested in investigating adrenal venous blood in the dog. At that time we didn't have a very sensitive colour reaction so we made our measurements according to the ultraviolet spectrum, as obtained from chromatographed fractions of the material and we felt that we could do quite a nice job with quantities of 1 μg. per ml. of blood. Certainly with 2 μg. per ml. of blood we could accurately measure either deoxycorticosterone, cortisone, or 17-hydroxycorticosterone added to blood. However, when we tried to apply these methods to peripheral blood we could not find measurable quantities, and therefore had to look for something a little more sensitive.

C. J. O. R. MORRIS: I think it would be very interesting to take fractions prepared in this way and run them on chromatograms rather as Bush has described.

In conclusion, our procedure, which is relatively rapid, appears to give a more reliable reflection of the clinical picture than urinary assays.

REFERENCES

[illegible] rinol., Supplement 8.
[illegible] J. Endocrinol. (in Press).
[illegible] rris, P., and WILLIAMS, D. C. (1951). Biochem J., 49, 434
NELSON, D H , SAMUELS, L. T., WILLARDSON, D. G., and TYLER, F. H. (1951). *J. clin. Endocrinol*, 9, 1021.
PASCHKIS, K E , CANTAROW, A., WALKLING, A. A., and BOYLE, D. (1950). *Endocrinology*, 47, 338
WICK, A N., HILLYARD, L., and MACKAY, E. M (1951). *Endocrinology*, 48, 137.

DISCUSSION

NELSON: Our experience with Cushing's disease coming to operation is very similar to that of Mr. Butt. We have three cases who before operation had very high values, estimated by our method, and who had very significant decreases in these values when most of one adrenal gland was removed. In the one case who had a tumour the values were much higher than those in the other two cases, but unfortunately we did not

[illegible]

the adrenal.

GADDUM: About how much higher are your results? Ten times?

NELSON: I believe you said 1–4 μg. per ml. plasma. Our values are about 5–20 per 100 ml So that's about 20 times.

CROOKE: I wonder if Dr. Morris has anything to say about the speci- [illegible]

hormone in the body And for that purpose the test has to be sensitive —I think that's the most important thing—and simple and specific and statistically sound, and a lot of other things that don't begin with "S," which were enumerated by Dr. Segaloff.

We have heard about a lot of new tests and it's interesting to consider how they stand up to these criteria. We heard of three newish methods of estimating thyrotrophin, all of which sounded interesting and promising We saw a picture of a chick which looked much more collaborative than you might have expected. It's difficult to know which of those methods is the best, and I suppose that time will show I don't think that I'm convinced that any of them is really sensitive enough to apply to patients and to estimate thyrotrophin in blood and urine We've heard nothing of the very sensitive method which has been described by D'Angelo. I think it may be that we will be driven

which were new to me and which I find difficult to fit into my knowledge of the subject The tests are certainly sensitive enough to detect gonadotrophins in patients, but they're not specific enough. We know that there are several gonadotrophins, and it's rather difficult to know which of them we're after

Perhaps prolactin is a gonadotrophin and perhaps it isn't. We heard some interesting facts about the prolactin effect of urine, and I hope that they will provide the basis of a way of studying the fate of prolactin in patients

CHAIRMAN'S CLOSING REMARKS

J. H GADDUM

I FEEL that we're on the fringe of an undiscovered country and that we're still examining our equipment and wondering whether it's going to do what we want it to do. What we want of our equipment depends very much on what we're going to use it for.

One important thing that people want to do is to fractionate hormones. The methods of assay which we use for that don't need to have any of the properties enumerated by Dr Segaloff at all, except that they

tion at all; I don't think that the people who isolated the œstrogens knew how to do a bioassay, but it didn't matter.

The people who fractionate hormones have been only too successful I used to think that there were only six in the anterior pituitary, and I've been rather disturbed by various things that I've heard at this

assay to samples of blood and urine, to find out what's happening to the

AUTHOR INDEX TO PAPERS

SUBJECT INDEX

patient is insensitive to it.

The tests are all rather elaborate, and I wonder what it will be like if we meet again five years hence and discuss the same subject. I suspect that only those of us who are chemists will be here at all, and they'll be incubating tissue slices and tissue mushes of various kinds, and then

is to eliminate the whole animal and replace it by something simpler and more constant in its responses

Printed by Richmond Hill ... Works, Ltd., Albert Road, Bournemouth